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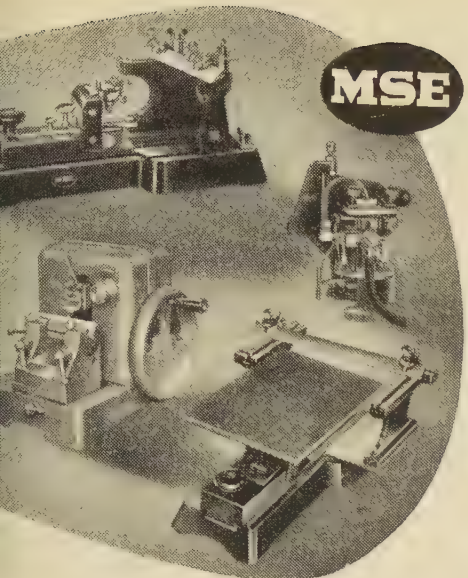

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'he Distribution of Esterase in the Nervous System and other Tissues of the Insect *Rhodnius prolixus*

By V. B. WIGGLESWORTH

(*From the Agricultural Research Council Unit of Insect Physiology, Department of Zoology, Cambridge*)

With one plate (fig. 1)

SUMMARY

'holinesterase in *Rhodnius* is limited to the neuropile, the nerve-roots, and the larger 'es. None is present in the axons; it seems to be confined to the interneuronal plasm, the product of the glial cells. The intensity of the reaction is greatest in the aptic regions and appears to be proportional to the amount of interneuronal erial.

'he ganglion cells contain traces of a non-specific esterase; and larger amounts of -specific esterase occur in the glial layer between the cells. A similar enzyme is ntiful within the perineurium cells.

Ton-specific esterases occur in many other tissues: salivary glands and alimentary al, pericardial cells, haemocytes, oenocytes, dermal glands and epidermal cells, n-cells and fat-body. Esterase is absent from the muscle endplates. The cyto-mic localization and the reaction of these enzymes to inhibitors are described.

n the fat-body, each droplet of fat has a single well-defined 'cap' of esterase, pre-ably lipase. It is suggested that this controls the transfer of triglycerides to and n the storage vacuoles. Esterase is not associated with the mitochondria; but there vidence that the enzyme may be disposed as fine filaments, particularly over the 'ace of the nucleus. Some of these widely distributed 'esterases' may be cathepsins.

'HE central nervous system of insects is rich in acetylcholine and cholin- esterase (Corteggiani and Serfaty, 1939); and the organophosphate ecticides are commonly believed to owe their toxicity to the inhibition of linesterase and the consequent accumulation of acetylcholine in excess adwick and Hill, 1947; Kearns, 1956; Spencer and O'Brien, 1957). The that acetylcholine is not toxic when injected into insects led to some doubt o whether this could be the primary mode of action of the organophosphate npounds, and it was suggested that the poisoning of other esterases might the cause of death (Hopf, 1954; Lord and Potter, 1954). More recently as been found that the sheath around the ganglia and nerves acts as a barrier h to acetylcholine (Twarog and Roeder, 1957) and to non-ionized inhibitors holinesterase (O'Brien, 1957). At the present time, therefore, it seems prob- that inhibition of cholinesterase is a main cause of the toxicity of the anophosphate compounds in insects as in mammals (Spencer and O'Brien, 7), although it does not necessarily follow that that is their sole mode of on.

ll this has led to an extensive study of the esterases of insects (Metcalf and ers, 1955, 1956), but little is known about the histological or cytological

distribution of these enzymes. The present work forms part of a reinvestiga-tion of the histology of the nervous system of the insect, which is in course of publication. It has been extended to include a few observations on the esterases in other tissues.

Material and Methods

All the observations have been made on the blood-sucking bug *Rhodnius prolixus* Stål.

Three methods for esterases have been used: (i) the acetylthiocholine method of Koelle as modified by Gomori (1952); (ii) the naphthol AS acetate method as described by Pearse (1954); (iii) the 5-bromoindoxyl acetate method of Holt and Withers (1952) as described by Pearse (1953), using copper sul-phate 10^{-3} M as oxidation catalyst. The indoxyl method proved to be the most generally useful. Various inhibitors were used in conjunction with these tests: eserine 10^{-5} M; paraoxon (E 600) 10^{-4} M; tetra-*iso*-propylpyrophosphor-amide (*iso*-OMPA) 10^{-4} M; 1:5-bis-(4-trimethylammonium-phenyl) pentane-3-one di-iodide (62.C.47 of Messrs. Burroughs and Wellcome) 10^{-4} M; and silver nitrate 10^{-2} M.

Whole organs or 15-μ frozen sections of tissues fixed for 24 h in cold neutral formaldehyde solution were placed in the substrate mixtures for 1–16 h at room temperature and ultimately mounted in Canada balsam, unstained or after staining with borax carmine or Bismarck brown. Other tests were made on fresh tissues using methods (ii) and (iii) with the reagents made up in isotonic saline. Exposure was then for 15–45 min and was followed by fixation in formaldehyde, sometimes preceded by brief fixation in osmium tetroxide.

Distribution of Esterase in the Nervous System

If the brain or the fused thoracic and abdominal ganglia, after fixation in cold neutral formaldehyde for 16 h, are washed in distilled water for half an hour and then placed for 6–24 h in the 5-bromoindoxyl acetate mixture, an intense blue coloration develops in the central parts of the ganglia, in the roots of the nerves as they pass through the layer of ganglion cells, and in the large nerves (fig. 1, A, D).

The intense reaction in the ganglia is confined to the neuropile. With the osmium ethyl gallate method of staining (Wigglesworth, 1957) the neuropile stains dark and the ganglionic layer is relatively pale. Fig. 1, B, E shows that the distribution of this staining agrees exactly with that of the esterase.

At a higher magnification of frozen sections of ganglia similarly treated it is seen that the axons are completely colourless; the reaction is confined to the material between the axons (figs. 1, F; 2, A). So that in cross-sections of the nerves or nerve-roots the axons are more or less ringed with the deposit of indigoid dye (compare Snell, 1957).

The structure of the neuropile will be described elsewhere (Wigglesworth, 1958) but it may be stated here that it consists of large and small axons and interaxonal material. Since the contents of the axons always give a negati

ıse reaction, the intensity of the blue coloration is proportional to the .nt of interaxonal material present (fig. 1, F). The most deeply coloured ns are those which stain most darkly with osmium ethyl gallate; in these ns the nerve-filaments are exceedingly fine and therefore there is rela- ' more interneuronal material, containing double membranes and mito- dria. This is believed to be the cytoplasm of the large glial cells. 'Synaptic les' within the nerve endings also contribute to the dark staining with ım and ethyl gallate.

addition to the neuropile there is a less intense reaction in the layer of lion cells and in the perineurium. The cytoplasm of the ganglion cells is ʼs completely negative (apart from some minute granules over the surface e nuclei, which will be discussed later). The deposit of indigoid dye is in orm of fine granules and thin sheets in the glial substance between the and along the nerve processes coming from them. In the case of very ganglion cells the glia is invaginated deeply into the cell-body in the of 'mesenteries'. The esterase reaction may sometimes extend in the of fine granules into these mesenteries.

the perineurium cells below the fibrous sheath of the ganglia (the peri- ıa) there are numerous fine granules of indigoid dye scattered through the olasm and concentrated around the nuclei.

the post-cerebral organs, there is a very weak reaction in the form of a cattered granules in the corpus cardiacum and a somewhat stronger reac- in the hypocerebral ganglion; the corpus allatum is negative.

the fresh unfixed ganglia and nerves are immersed in the 5-bromoindoxyl te mixture in isotonic saline they become blue within a few minutes. But after one hour the colour is almost wholly confined to the perineurium; is usually no reaction within the neuropile of the ganglia or between the s in the nerves (fig. 2, B). In the perineurium cells there are fine granules, scattered through the cytoplasm but most concentrated over the surface e nucleus, so that this appears outlined in blue. It would appear that r these conditions the substrate material will penetrate the perilemma to the perineurium cells but it does not reach the enzymes in the deeper of the nerves or ganglia. But if the ganglia or nerves are cut across before rsion, the enzymes show the same distribution as in the fixed material. r these conditions the indigoid dye is deposited in very small granules in europile.

nilar results are obtained with acetylthiocholine. This is far less penetrat- nan bromoindoxyl acetate and even in formaldehyde-fixed ganglia the on is almost limited to the perineurium; and when the ganglion is cut gh the reaction is confined to the exposed surface. But the distribution described: most intense in the neuropile and between the axons in the s, but positive also in the perineurium cells and between the ganglion In 15-μ sections of the ganglia the weak reaction in the perineurium and ar layers is almost invisible and the esterase appears confined to the pile.

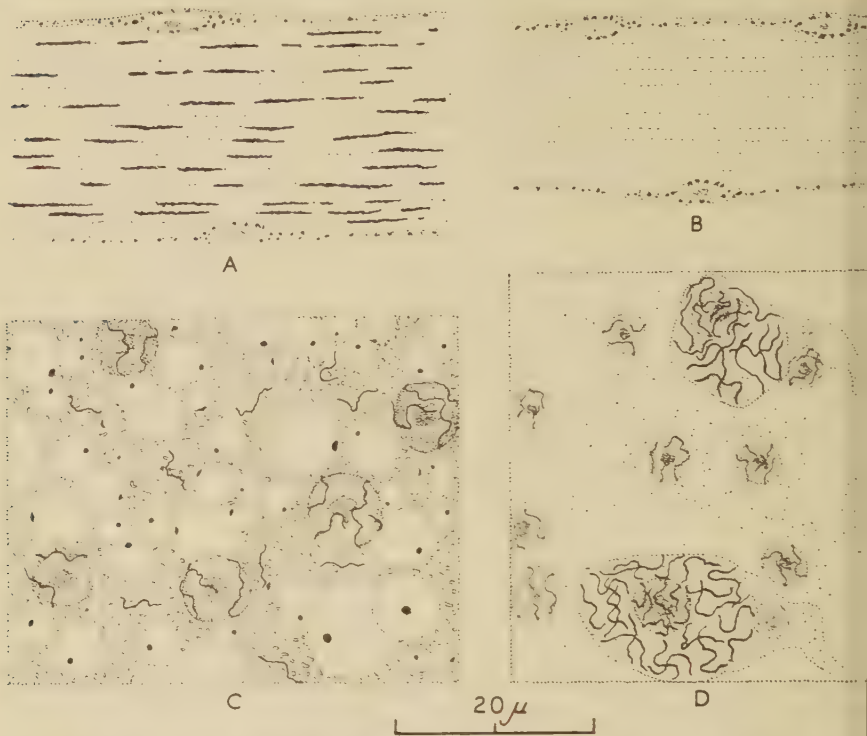

FIG. 2. A, distribution of esterase in perineurium and between axons of nerve. 5-bro indoxyl acetate after formaldehyde fixation. B, nerve after exposure to 5-bromoindoxyl ace in the fresh state. Esterase reaction limited to the perineurium cells. C, whole mount of body after exposure to 5-bromoindoxyl acetate in the fresh state. Esterase reaction in form 'caps' on each fat vacuole and fine filamants over the surface of the nuclei and among mitochrondria. D, epidermis treated as C. Esterase reaction in form of very fine filam over the nuclei of the epidermal cells and larger filaments throughout the cytoplasm of dermal glands.

THE ACTION OF INHIBITORS ON ESTERASE IN THE NERVOUS SYSTEM

Identification of true cholinesterase rests on the effect of selective inh tors. Whole ganglia or frozen sections, after formaldehyde fixation, w immersed in the solution of inhibitor for half an hour and then transfer to the substrate mixture to which more inhibitor had been added. F inhibitors were tested on the central nervous system, using 5-bromoindo acetate as substrate. The results were confirmed with acetylthiocholine.

(i) Eserine 10^{-5} M is an inhibitor of the cholinesterase group of enzymes completely inhibits the intense reaction in the neuropile and between the ax in the nerves, leaving only some scattered granules of dye. But the reac in the perineurium and between the ganglion cells is not affected, so that the neuropile appears paler than the rest of the ganglion (fig. 1, C).

(ii) Tetra-*iso*-propylpyrophosphoramide (*iso*-OMPA) 10^{-4} M is a spe inhibitor for the 'pseudo-cholinesterase' which occurs in the central nerv

tem of mammals (Pepler and Pearse, 1957). It has no apparent effect on the erases in the neuropile, perineurium, or ganglion layer in *Rhodnius*.

iii) 1 : 5-bis-(4-trimethylammonium-phenyl)pentane-3-one di-iodide .C.47) 10^{-4} M is a specific inhibitor of the true acetylcholinesterase of mmals (Pepler and Pearse, 1957). In the ganglia of *Rhodnius* it acts just like rine: it inhibits the reaction in the neuropile and between the nerve axons t has no effect on the esterase of the perineurium and cellular layer.

iv) *oo*-diethyl *o*-*p*-nitrophenyl phosphate (paraoxon; E 600) 10^{-4} M inhibits uch wider range of esterases in mammals (Aldridge, 1953). In *Rhodnius* it npletely inhibits the reaction in the neuropile, and very largely, but not npletely, inhibits that in the remainder of the ganglion.

From these results it appears that the abundant enzyme in the neuropile l between the nerve axons is a specific acetylcholinesterase. There is no dence of an enzyme with properties resembling those of 'pseudo-cholin-erase' as found in mammals. The esterase between the ganglion cells and the perineurium cells clearly does not belong to the cholinesterase group though it does appear capable of hydrolysing acetylthiocholine to some all extent).

Esterase in Other Tissues

Enzymes hydrolysing acetylthiocholine are confined to the nervous system Rhodnius but enzymes hydrolyzing 5-bromoindoxyl acetate and naphthol acetate are widely distributed in other organs and tissues. They have been died in fresh and formaldehyde-fixed material. The 5-bromoindoxyl ace-e penetrates into the cells very readily and gives better cytological results. If the recently fed 4th-stage larva of *Rhodnius* is slit along each side of the domen and the tergites and sternites, after separation from the alimentary al, are immersed for 30 min in the isotonic substrate without previous tion, the indigoid dye is deposited in many tissues.

Fat-body. The lace-like fat-body becomes blue within ten minutes. Micro-pic examination of the fresh tissue shows that each fat droplet, from the allest (measuring one or two microns in diameter) to the largest (measuring μ or more) has a small point or cap which is the site of the reaction (figs. 1, 2, C). If the tissue is fixed in formaldehyde and mounted in Canada balsam an be seen that in addition to the conspicuous 'caps' on each fat vacuole, re are exceedingly fine blue filaments applied to the surface of the nuclei l a few similar filaments and fine granules elsewhere in the cytoplasm s. 2, C; 1, L). When examined fresh the 'caps' on the fat droplets are even l sharply defined; after fixation in formaldehyde they are somewhat nkled and distorted; after fixation in Carnoy they break up into irregular mps of dye in a pale blue ground substance.

f the fat-body, after exposure to the substrate, is fixed for 2 min in 1% nium tetroxide, and then for 5 min in saturated picric acid, kept in Far-ts's medium for several days (so that the fat droplets become pale again) l then stained with borax carmine and Sudan III and mounted in Farrants's

medium, it is possible to see the mitochondria very faintly, as well as th
stained fat droplets and nuclei. It is then apparent that the fine blue filamen
are much more slender than the mitochondria among which they lie. In the
osmium-fixed preparations the 'caps' on the fat droplets retain the sharp ou
line which they have in fresh preparations.

After formaldehyde fixation the same reactions can be obtained but t
'caps' on the fat droplets are irregular in outline and partially broken up, a
there are no uniform filaments on the surface of the nuclei, but only rows
granules.

With naphthol AS acetate identical 'caps' appear on many of the fat dropl
in fresh and fixed preparations (fig. 1, G). But no filaments can be seen
either.

The salivary glands and alimentary canal give a strongly positive reacti
in fresh and fixed preparations. This takes the form of very fine dispers
granules which are most densely concentrated on the surface of the nuclei.

The Malpighian tubules give an intense reaction, which appears in the fre
material within 2 min after immersion, in the form of fine granules dispers
throughout the cell, but concentrated on the surface of the nuclei, and a di
fuse blue coloration of the striated border.

Ovary. The rudiment of the ovary as it exists in the 4th-stage larva rea
strongly in the fresh state. The cells of the developing duct and the th
mantle of mesodermal cells covering the germ-cells are negative; but the ger
cells themselves contain great numbers of blue filaments. These are conce
trated around the nuclei and in some of the cells are confined to the surf
of the nuclear membrane (fig. 1, K).

The epidermis and dermal glands give a positive reaction (fig. 2, D). In t
ordinary epidermal cells there are just a few very slender filaments applied
the surface of the nuclei. In the principal gland-cell of the dermal glands t
filaments are somewhat stouter and besides being applied to the surface of t
nucleus they run in all directions in the substance of the cell. They hav
more or less uniform size and appearance in all the dermal glands.

Pericardial cells. The reaction in these cells is particularly conspicuous af
formaldehyde fixation. It takes the form of very fine granules evenly disper
among the rounded inclusions in these cells, with some concentration arou
the nuclei.

Haemocytes and oenocytes. The haemocytes contain fine granules, of
concentrated around the nucleus. In the oenocytes the reaction is mostly co
fined to filaments which are rather denser over the nuclear membrane but
also dispersed throughout the cytoplasm (fig. 1, J). The whole reaction is w
in the newly fed insect. But as growth proceeds and the oenocytes enlarge a
become charged with their lipoprotein product (Wigglesworth, 1933, 19
the reaction increases in intensity, so that by seven days after feeding (in
4th-stage larva) the oenocytes stand out conspicuously by reason of their b
colour, and by this time the whole cytoplasm is filled with blue granules a
filaments.

Muscles. The muscles are completely non-reactive apart from the fine ranules and filaments which sometimes develop on the surface of the nuclei ı the fresh preparations. There is no sign of any positive reaction in the ıuscle endplates, where cholinesterase is so conspicuous in vertebrates Chessick, 1954; Couteaux, 1955; Holt, 1954).

In most of these tissues, but particularly in the fat-body and oenocytes, the ıtensity of the reaction increases during the moulting cycle when reserves of ll kinds are being deposited in the cells.

The Action of Inhibitors on the Esterases of Non-nervous Tissues

Eserine, *iso*-OMPA and 62.C.47, inhibitors of the cholinesterase group of nzymes, have no apparent effect on any of the enzyme sites described in the ast section.

Paraoxon (E 600) at 10^{-4} M, besides causing complete inhibition of the holinesterase of the nervous system, brings about a partial inhibition of nany of the other enzymes, such as that in the Malpighian tubules, alimentary anal, pericardial cells, and fat-body. But in no case was this inhibition omplete.

On the other hand, silver nitrate 10^{-2} M causes complete inhibition of the sterases of the fat-body, gut, and Malpighian tubules, but fails to inhibit that n the pericardial cells.

Discussion

Esterase in the nervous system. The three histochemical tests employed learly reveal a number of quite different 'esterases'. The only enzyme which s inhibited by eserine and by 62.C.47 is that which occurs so abundantly etween the axons in the peripheral nerves and nerve-tracts in the ganglia, nd particularly in the neuropile. There is little doubt that this enzyme is a rue cholinesterase.

In the nerves this cholinesterase is located in the interaxonal material which is the product of the neuroglial cells; the axon contents seem lways to be negative. In the neuropile the reaction is most intense in those egions where the interneuronal material is most abundant. In these places as studied with the electron microscope) the axons and dendrites are exceedngly fine and the material between them is filled with double membranes 'endoplasmic reticulum') which are largely responsible for the deep staining with osmium and ethyl gallate. This cytoplasm, which also contains mitochondria, appears to be the product of the neuroglial cells and it seems likely hat this is the site of the cholinesterase.

In the rat and other mammals (Koelle, 1954; Giacobini, 1956; Pepler and Pearse, 1957) the specific cholinesterase occurs within the cell-bodies of the neurones and along the axons and dendrites of many groups of neurones; the non-specific cholinesterase in neurones and in certain of the gliocytes. The conditions in *Rhodnius* resemble more closely those described in the frog. In

the frog, Shen, Greenfield, and Boell (1955) found that in all cases the enzyme-rich regions are centres with dense and elaborate interneuronal connexions, most of which (apart from motor neurones) contain few cells. They concluded that the enzyme is associated primarily with synaptic connexions and not with cell-bodies. In the optic lobes of the frog cholinesterase appears as the synapses become mature (Boell, Greenfield, and Shen, 1955).

The contents of many of the terminal axons and dendrites in the neuropile of *Rhodnius* also stain with osmium and ethyl gallate (whereas throughout most of their course the axons are almost unstained, apart from the numerous mitochondria which they contain). As seen with the electron microscope, this dark staining is due to the abundant spherical inclusions about 250 Å in diameter, the so-called 'synaptic vesicles'. In vertebrates these are commonly regarded as the site of acetylcholine production.

When fragments of the ganglia in the fresh state are immersed in the indoxyl reagent the deposit of dye appears in very fine granules. It is impossible to say whether such granules represent preformed organelles; it is perhaps safer to regard them as chance deposits of dye. Smallman and Wolfe (1956) and Wolfe and Smallman (1956) found that in homogenates of the central nervous system of insects, cholinesterase is present in both soluble and particulate fractions. Metcalf and others (1956) showed that the cholinesterase was largely particulate, the 'aromatic esterase' largely soluble.

Muscle endplates. The sole-plate of vertebrate muscle is the site of an active esterase which is thought to be concerned with the rapid breakdown of the transmitter substance, acetylcholine (Couteaux, 1955; Chessick, 1954). In insects the nature of the transmitter substance is unknown; the endplates in *Rhodnius* appear not to be the site of any esterase.

Esterase in the fat-body. It has always been difficult to picture how the transfer of fatty material to and from the individual fat droplets is brought about. The present observations show that each droplet has its own sharply localized 'cap' of esterase.

This cap has an identical appearance whether revealed with 5-bromoindoxyl acetate or with napthol AS acetate. The enzyme is not inhibited by 10^{-4} M eserine and is only partially inhibited by 10^{-4} M E 600 (paraoxon). There can be little doubt that this is a genuinely localized enzyme site and it seems most probable that the enzyme in question is a true lipase. Lipase of the pancreas will hydrolyse 5-bromoindoxyl acetate (Pearson and Defendi, 1957) and is not inhibited by E 600 (Aldridge, 1954). It is suggested that at these enzyme sites, localized reversible hydrolysis of triglycerides controls the transfer of fat to and from the storage vacuoles.

The significance of the filaments on the surface of the nuclei and among the mitochondria is more doubtful. Are these filaments preformed structures, the site of an 'esterase', or are they mere deposits of indigoid dye that happen to have assumed a filamentous form? It is quite common to see the indigoid dye separating out in crystalline filaments which are certainly artifacts; but that does not prove that the filaments under discussion are also artifacts. The ques-

must remain undecided, but the evidence in support of their being pre-
ned structures is as follows:

) In the fat-body they have a uniform thickness, which is the same in every
.

ii) They have this even appearance only when the substrate is applied to sh material. In fixed material the reaction product appears in the form of nules; and, as we have seen, the caps of esterase on the fat droplets like-e tend to be broken up in fixed material.

(iii) Filaments applied to the surface of the nucleus occur in many cells ated in the fresh state. These filaments have a characteristic size in each e of cell; they are almost invisibly fine (probably 0·1 μ thick, or less) in the dermal cells, larger in the fat-body cells, and larger still in the dermal nds and in the germ-cells of the developing ovary. On the other hand, they not occur in all cells. For example, in the Malpighian tubules there are all granules scattered through the cell; there are denser around the nuclear mbrane, but filaments are absent.

One can do no more than speculate on the nature of the enzyme represented the filaments. It might be a peptidase connected with protein synthesis; , as Pepler and Pearse (1957) and Hess and Pearse (1958) have pointed , 5-bromoindoxyl acetate is readily hydrolysed by intracellular cathepsins, l these enzymes are resistant to inhibition by E 600 but are completely in-bited by silver nitrate. Or it might be concerned in phospholipid metabolism.

The same uncertainty surrounds all the other sites of 'esterase' action that ve been described. It is interesting to note the abundant filaments in the nocytes and in the germ cells of the ovary where active synthesis of protein d lipid is in progress.

The abundant esterase in the pericardial cells is of interest, for the resis-ace of this enzyme to inhibition by silver nitrate suggests that it is of a ferent nature from the other enzymes described. The pericardial cells ively segregate colloidal particles of dyes, haemoglobin, and other foreign tter introduced into the blood. They are often compared with the reticulo-dothelial system of mammals; otherwise their function is not known.

I am indebted to Dr. A. G. Everson Pearse for permitting me to see the per by Dr. R. Hess and himself before publication and for the supply of bromoindoxyl acetate, and to Dr. D. Prescott of the Wellcome Foundation d., for a sample of 62.C.47.

POSTSCRIPT

While this paper was a proof, Holt and Withers (1958), using an improved bstrate (5-bromo-4-chlorindoxyl acetate) on fat cells of the rat, have ported stained organelles in the cytoplasm close to the nuclei.

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FIG. 1 (plate). A, whole ganglion (fused meso- and metathoracic and abdominal gangl of 4th-stage larva showing esterase reaction in neuropile, nerve roots, and nerves. 5-brom indoxyl acetate.

B, horizontal section of ganglion showing dark staining of neuropile with osmium and eth gallate.

C, esterase reaction in whole ganglion in the presence of eserine 10^{-5} M. The reaction the neuropile and nerves has been inhibited. 5-bromoindoxyl acetate.

D, whole brain of 4th-stage larva treated as A. Esterase reaction confined to neuropile.

E, horizontal section of brain showing staining of neuropile with osmium and ethyl galla

F, frozen section of ganglion fixed in formaldehyde solution, showing absence of ester in the axons, positive reaction between the axons, and intense reaction in the synaptic regio 5-bromoindoxyl acetate.

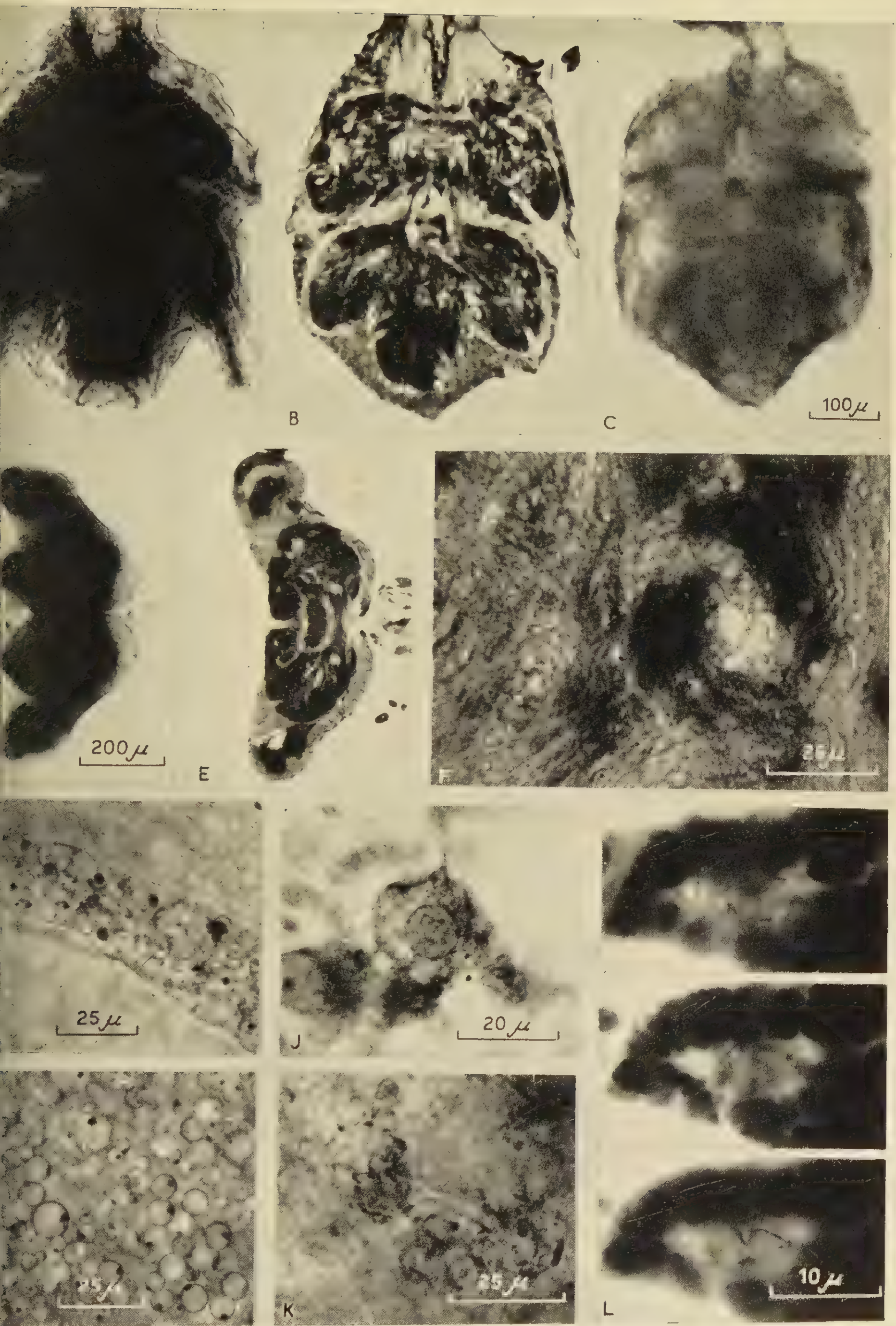
G, fat-body mounted whole; esterase confined to a small area on each fat droplet. Napht AS acetate after formaldehyde fixation.

H, fresh unfixed fat-body showing a sharply defined 'cap' of esterase on each fat drop 5-bromoindoxyl acetate.

J, esterase in the form of filaments on the nucleus and in the cytoplasm of oenocyt 5-bromoindoxyl acetate.

K, three large germ-cell nuclei in ovary rudiment, showing esterase in the form of filame over the surface. 5-bromoindoxyl acetate.

L, a single nucleus in the fat-body after exposure in the fresh state of 5-bromoindo acetate, showing filaments close to the nuclear membrane. Above, lower surface of nucle middle, optical section of nucleus; below, upper surface of nucleus. The surrounding fat h been darkened by osmium.



V. B. WIGGLESWORTH

Dimethyl Hydantoin Formaldehyde: A new Water-soluble Resin for Use as a Mounting Medium

By H. F. STEEDMAN

(*From the Department of Zoology, University of Glasgow*)

Summary

Dimethyl hydantoin formaldehyde resin dissolved in water or in 70 ml ethyl alcohol + 30 ml water is put forward as a solution suitable for the mounting of insects, small crustacea, &c. Its chief advantage over other water-soluble resins is its high solubility in water, its excellent adhesion to glass, its low viscosity even in high percentage solutions, and its hardness on drying.

Physical and Chemical Data

Colour: water-white to pale amber.
Odour: faint.
Form: brittle lumps.
Molecular weight: 240–300.
Initial softening point: 59° C–80° C.
Free formaldehyde: approximately 0·3% in aqueous solution.
Solubility in water: solutions up to 80% may be prepared with ease and filtered.
pH of solution: 6·5–7·5.
Soluble in methanol, ethyl acetate, methyl ethyl ketone, methylene chloride.
Insoluble in xylene, benzene, diethyl ether, carbon tetrachloride, trichlorethylene.
Supplied by Rex Campbell, 7 Idol Lane, London, E.C.3.

Histological Data

Stained sections: unsuitable because the resin will ultimately wash out the stain.

Whole mounts: excellent for all arthropods, turbellaria, teased materials such as muscle fibres stained with gold chloride, &c. Such specimens may be mounted directly from water or from alcohol solutions, or from formaldehyde solutions.

Strength of solution: the resin may be dissolved in either pure water or in water and alcohol solutions. The pure water solutions show a tendency to develop moulds and the solution recommended is therefore made up with 70 ml ethyl alcohol + 30 ml water, or in phenol solution (5% aqueous).

Refractive index:	DMHF resin	70 g	R.I. 1·457.
	Distilled water	30 ml	
	DMHF resin	70 g	R.I. 1·466.
	70% alcohol	30 ml	
	Dry resin		R.I. 1·54.

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Effect on tissues: solutions of dimethyl hydantoin formaldehyde keep tissues soft. The following solution may be used in tubes or bottles as a preserving fluid for insects, &c., until it is convenient to mount them on slides:

dimethyl hydantoin formaldehyde	50 g
ethyl alcohol, 70%	50 ml

Penetration of tissues: a whole *Daphnia* mounted from water is translucent within 10 h.

Drying of mounted preparations: this may be done by leaving the slides on the bench at room temperature, or by drying in an oven at about 40° C for 3 or 4 days. Heating above 40° C produces bubbles in the resin. With large whole mounts the resin remains soft for some time.

Tendency to retain water: the solutions dry to a hard mass which retains about 3–4% water. This prevents the resin becoming brittle and flaking away from the microscope slide.

Preparation of Solutions

1. Grind the resin to a powder. A mincing machine such as is used for household purposes is most suitable for bulk work.

2. Take 70 or 80 g of the ground resin as required and add 30 ml or 20 ml of distilled water, or of 70% alcohol. Shake at times for 3–5 days until the resin is dissolved.

3. Filter through a Barcham Green 904 filter paper. The 70% resin solution will go through the paper quite readily.

4. Refilter. The total resin loss as a result of filtering is 6%.

5. If the solution is purely aqueous, add sufficient phenol to make a 5% solution. This is not necessary when the resin is dissolved in 70% alcohol.

For small thin mounts the 70% resin solution is recommended: for large thick specimens the 80% solution.

I am happy to acknowledge skilled technical assistance from Mr. Peter Anderson.

Unmasking of Sudanophil Lipid in the Testis of the House-cricket, *Acheta domesticus*

By BLANCHE-P. CLAYTON

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

Summary

1. When the testis of *Acheta domesticus* is fixed in Flemming's fluid and embedded in gelatine, Sudan black reveals scarcely any lipid in the externum of the acroblast.

2. It can be shown that the externum of the acroblast contains much lipid in a masked (bound) form.

3. Experiments were performed to find what substances were effective, after Flemming fixation, in unmasking the lipid of the externum and thus making it colourable by Sudan black.

4. The following substances were found to act as unmasking agents: ethanol, dioxane, phenol, resorcinol, hydroquinone, pyrogallol, tannic acid.

5. Under the conditions of the experiment, the most effective unmasking agents were 90% ethanol and 5% hydroquinone.

Introduction

WHILE a cytochemical study of spermatogenesis in the house-cricket, *Acheta domesticus* (L.), was being carried out, it was noticed that certain cytoplasmic inclusions (pro-acroblast, acroblast, mitochondria) were almost entirely negative (absence of blue to blue-black coloration) to Sudan black. This negative result was obtained after the testes had been subjected to fixation by strong Flemming's fluid, washing, and gelatine embedding. On the other hand, it was found that the same inclusions were strongly positive to Sudan black if paraffin sections were used. The fixed material was passed through the alcohols and chloroform into paraffin; the wax was removed from the sections by soaking for 10 min in dioxane at 60°.

The almost negative results obtained by using gelatine-embedded tissue, and the very positive results obtained after embedding in paraffin, suggested that, in effect, one or other of the various compounds employed after fixation was acting as a lipid 'unmasking' agent: the lipid, having been in some manner split from the associated protein, was now rendered sensitive to detection by Sudan black.

For the above reasons, it was thought worth while to make a further, more detailed study in order to find out what compound (or compounds) was acting in this manner.

In this connexion it was considered that, as ethyl alcohol is known to act as an unmasker when used simultaneously with the fixative (Ciaccio, 1926), it was also acting in this capacity after (Flemming) fixation.

Furthermore, as phenol is known to be a lipid unmasker when used after

fixation (Ciaccio, 1926), certain other phenolic compounds (hydroquinone, pyrogallol, resorcinol, and tannic acid) were chosen for experimentation. Finally, as dioxane had been used, in the initial work, for dewaxing sections preparatory to staining, it was also included.

As a test object for the demonstration of unmasked lipid, a cytoplasmic inclusion, the acroblast, was chosen. This structure was selected because the chemistry of its constituents had already been studied fairly extensively by Clayton, Deutsch, and Jordan-Luke (1958). This work demonstrates that the acroblast consists of an outer and an inner part. The outer, or externum, appears to be made up of parallel lamellae, arranged (in electron micrographs) in the form of a horseshoe. It gives a reaction (among others) for a lipid. The externum is used in the present study to assess the effect of unmasking agents. As the inner part, the internum, appears almost structureless (by light microscopy) and gives only a very feeble (or no) reaction for lipid, reference to it is omitted.

Materials and Methods

Testes of the house-cricket were rapidly dissected out of lightly anaesthetized animals and were fixed in Flemming's fluid; thereafter, they were washed and (usually) prior to gelatine embedding, were subjected to the treatments listed under tables 1 and 2 in the appendix. With reference to these experiments, it is to be understood that each one is concerned with one piece of tissue only: each experiment was self-contained and was planned to demonstrate the effect (in lipid unmasking) of one compound only. Furthermore, for the sake of clarity in interpretation of the tables, some notes are added below.

(1) Both ethyl alcohol (of various concentrations) and dioxane were used on the tissue after fixation and washing and before embedding.

(2) All the other compounds (hydroquinone, phenol, pyrogallol, resorcinol, and tannic acid) were tested on gelatine sections.

(3) The period in pyrogallol was restricted to one half-hour, because, after this time, a general darkening of the tissue impeded interpretation of the results obtained by Sudan black.

(4) A control was carried out by applying the Sudan black technique to tissue fixed in Flemming's fluid but not subjected to any other unmasking treatment.

(5) In order to confirm the original observation—sudanophilia after Flemming fixation and paraffin embedding—the Sudan black technique was again carried out on tissue fixed and embedded in this manner. The results were again positive.

(6) The Sudan black, used in all experiments, was prepared and employed according to Baker (1949). Solutions not more than 3 weeks old were used. For this technique, two periods were chosen: $2\frac{1}{2}$ min at room temperature and 10 min at 60° C.

(7) Sudan black lability was tested by placing sections coloured by Sudan black in 70% ethanol for $\frac{1}{2}$ h and noting whether the colour disappeared.

Results

The results of the individual experiments are listed under tables 1 (effect compounds used as lipid unmasking agents) and 2 (effect of lipid extraction sudanophilia) (see the appendix). An examination of these tables will show following facts.

(1) All the compounds tested as unmasking agents give sudanophilia to a ater or less degree. Both ethanol (90%) and hydroquinone (5%) afford ults optimal in effect and clearest in interpretation. With respect to the er point, dioxane, pyrogallol, and tannin all tend to give a general cyto-smic darkening.

(2) In the control (table 1) the results are almost entirely negative. The ak sudanophilia shown by the externum of the acroblast may well be due the unmasking action of the ethanol (70%) used as the solvent for the Sudan ck.

(3) Extraction by xylene slightly inhibits the sudanophilia and that by ridine abolishes it, perhaps by solution of the lipid in the unmasking.

Discussion

Ciaccio (1926) long ago demonstrated that, among other substances, both anol and phenol could act as lipid unmasking agents; of these two com-unds, he used ethanol simultaneously with the fixative and phenol after it. ntil very recently no other workers appear to have carried Ciaccio's experi-nts further. Lately, however, Gupta (1958) has shown that phenol also ts as an unmasking agent after fixation. Bradbury and Clayton (1958) have so given evidence that Flemming's fluid itself, without any post-fixation atment, acts very efficiently on masked lipids in mammalian tissue (basal gion of the acinar cells of the mouse pancreas).

The present study supports both the unmasking techniques mentioned ove, and, in addition, gives further evidence of compounds, other than hanol and phenol, acting after fixation.

Until recently, very little was known about the structure of lipoproteins d, consequently, about the bonding between the components (Macheboeuf, 38; Dawson, 1957); lately, however, it has been suggested (Engström and inean, 1958) that the protein is almost certainly linked with the polar groups the ionic lipids (lecithin, cephalin, &c.). But, even though this fact seems w fairly well established, our knowledge, in general, concerning these com-ounds (lipoproteins) is still too fragmentary to allow any serious attempt at plaining the actual mechanism whereby lipid unmasking agents disrupt the poprotein bonding.

As a result of the present study, it is suggested that ethyl alcohol at a con-entration of 90% be employed as a convenient and simple method for lipid nmasking. Moreover, this method is in accordance with that long used in iochemical extraction of lipoproteins (Dawson, 1957); even though, in ytochemistry, it is employed after fixation.

The writer wishes to acknowledge with pleasure much valuable advice an criticism, which, in the course of this work, arose during discussions wi Dr. J. R. Baker, F.R.S., and also suggestions and technical help contribut by Mrs. A. Przelecka and by Mrs. Barbara Jordan-Luke. She also wishes thank Professor A. C. Hardy, F.R.S., in whose department this work w carried out.

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APPENDIX

Table 1

A summary of the effects of compounds used as unmasking agents (when employ after fixation) on bound lipids in the acroblast of the spermatid of the hous cricket, Acheta domesticus

Treatment subsequent to fixation and prior to the use of Sudan black	*Sudan black technique*	*Degree of sudanophilia shown by the externum of the acroblast*
Ethanol, 50%	2½ min	O
6 h	10 min, 60° C	+ + to + + +
Ethanol, 70%	2½ min	+ to + +
4 h	10 min, 60° C	+ + +
Ethanol, 50%, 70%, 50%	2½ min	+ +
6 h	10 min, 60° C	+ + +
Ethanol, 90%	2½ min	+ + to + + +
6 h	10 min, 60° C	+ + +
Ethanol, 100%	2½ min	− to + +
6 h	10 min, 60° C	+ + +
Dioxane, 100%	2½ min	+ + +
½ h	10 min, 60° C	+ + +
Hydroquinone, 5%	2½ min	− to + +
1 h, 60° C	10 min, 60° C	+ + +
Phenol, 1%	2½ min	+ +
24 h	10 min, 60° C	+ + +
Pyrogallol, 5%	2½ min	+
½ h, 60° C	10 min, 60° C	+ + to + + +
Resorcinol, 5%	2½ min	+
1 h, 60° C	10 min, 60° C	+ +
Tannic acid, 20%	2½ min	+
	10 min, 60° C	+ + to + + +
No treatment	2½ min	O
	10 min, 60° C	+

Note: the treatment above designated as 'ethanol, 100%' actually comprised passage of th tissue up and down alcoholic solutions of the following concentrations: 50%, 70%, 80% 94%, 100%, 94%, 80%, 70%, 50%.

TABLE 2

summary of the effects of extraction by xylene and pyridine on lipids unmasked by ethyl alcohol

Treatment subsequent to fixation and prior to the use of Sudan black	*Sudan black technique*	*Degree of sudanophilia shown by the externum of the acroblast*
lcohol series,	$2\frac{1}{2}$ min	+
xylene, 4 h	10 min, 60° C	+
lcohol series,	$2\frac{1}{2}$ min	+
xylene (boiling), 4 h	10 min, 60° C	+
lcohol series,	$2\frac{1}{2}$ min	O
pyridine, 60° C, 15 h	10 min, 60° C	O

KEY TO TABLES 1 AND 2. +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative.

ote on the Use of Bromophenol Blue for the Histochemical Recognition of Protein

By JOHN R. BAKER

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

SUMMARY

There is not sufficient evidence to prove that bromophenol blue is a reliable histo-emical reagent for the recognition of protein. It is a powerful acid dye, capable of aking direct links with basic groups in tissue-constituents (and perhaps also with rtain acidic groups, through mercury).

BROMOPHENOL blue has recently come into general use as a histo-chemical reagent for the recognition of protein. It is the purpose of this ote to inquire whether a microscopical object that is coloured by this dye cessarily contains protein.

Bromophenol blue is an acid phthalein dye, commonly used as a pH dicator. It was used by Durrum (1950) for colouring proteins in paper-ectrophoresis. Durrum dissolved it at 0·1% in a saturated solution of ercuric chloride in 95% ethanol. The purpose of the protein coagulants iercuric chloride and ethanol) was to immobilize the proteins on the paper. urrum made no claim that the dye provided a specific test for proteins, but thing else in human blood-serum was coloured by it, under the conditions his experiments. The various proteins of serum were similarly coloured by and were distinguished by the speeds at which they moved in electro-ioresis.

Kunkel and Tiselius (1951) used bromophenol blue in a similar way. They metimes dissolved the dye in an aqueous solution of mercuric chloride, idified by acetic acid. They brought forward no evidence that the dye is ecific for proteins, and indeed they noted that both dextran and paper can certain circumstances be coloured by it. Cremer and Tiselius (1950) gave special method for removing the colour from paper while leaving it in otein.

Geschwind and Li (1952) showed that certain amino-acids and peptides on per chromatograms give a blue coloration with bromophenol blue. The sults of their experiments did not depend primarily on the ability or in-ility of the dye to colour these substances, but rather on the capacity of ercuric chloride to fix the dyed substance on the paper, and thus prevent removal by solution during the final rinsing in water. This would not cessarily be relevant in histochemical technique, for the amino-acid or ptide might have been rendered insoluble by the fixative used, and sub-quently coloured by the dye.

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Mazia, Brewer, and Alfert (1953) showed that the tissues of animals, fixe in routine fixatives and sectioned, could be strongly dyed by Durrum's solu tion. They also showed that starch and glycogen had no affinity for the dy and DNA and cholesterol very little. These results were to be expected, sin bromophenol blue is an acid dye.

The affinities of bromophenol blue, as reported above, do not qualify th dye to act as a reliable reagent for the histochemical recognition of protei. Such qualification could only result from the test of a wide variety of lipid carbohydrates, and other tissue-constituents.

In the present state of knowledge it seems safest to regard bromophen blue as a powerful acid dye, capable of making direct links with basic group in tissue-constituents, and perhaps also (as Mazia and his colleagues sugges with certain acidic groups, through mercury. Interesting results can b obtained by using the dye with and without mercury, on the same tissue; bu no certainty can be reached as to the chemical composition of the substanc coloured, unless reliable evidence is available from other sources.

Azocarmine B has been used to visualize proteins in paper-electrophores (Turba and Enenkel, 1950), but this does not mean that azocarmine is a sui able substance for the histochemical recognition of protein.

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The Uptake of Intravital Dyes by the Testis

By J. H. GRANT

(*From the Department of Anatomy, University of Liverpool*)

With one plate (fig. 1)

Summary

The vital staining reactions of the normal and ischaemic testis were investigated in o rats by the use of varying amounts of trypan blue and Unna's polychrome methy-ne blue. The experiments showed that it was possible to distinguish both true and eticulo-endothelial interstitial cell types. The implications of this subdivision are iscussed and their importance in relation to neoplasms of the Leydig cells stressed. 'he distribution of mast cells in the testis is described.

Introduction

BOUFFARD (1906) was the first to describe the histological appearance of the presence of intravital dyes in the testis, but he failed to state vhether they were to be found in all the intertubular cells, or merely limited o a particular group of them.

Goldmann (1909) not only gave a description of the cytological characteristics nd situation of the macrophage cell in the testis, but was also the first nvestigator to recognize their widespread distribution in the body. He ssigned a trophic role to them, claiming that they passed across the basement nembrane of the seminiferous tubules from the intertubular tissue, and, aving shed their granules of dye, united with either Sertoli cells or sper-natids. These conclusions were in agreement with earlier work carried out by 'lato (1896), Lenhossek (1897), Barbeleben (1897), and Friedmann (1898). Most of the later authors, except Mason and Shaver (1952), denied that migra-ion of the macrophages took place under normal conditions; their opinion vas based on indirect evidence since they did not observe the movements of hese cells.

Research into the staining of intertubular cells by intravital dyes has created onfusion as to whether the Leydig cells possess 'phagocytic' properties. Evans and Schulemann (1914), Esaki (1928), and Van Os and Ruyter (1939) elieved that the interstitial cells of Leydig were able to 'phagocytose' these yes during all, or at least certain, phases of their life cycle. Other investigators eld the opposite view that the interstitial cells were wholly incapable of this ctivity (Addison and Thorington, 1917; Bratianu, 1930*a*; Guerriero, 1930; tein, 1931). Whilst subscribing to the former opinion Takamori (1921) elieved that intravital dyes coloured Leydig cells lightly after the admini-tration of large amounts of these substances. This conclusion was criticized y Elek (1924), Natali (1925), Cannon and others (1929), and Cappell (1929) n the grounds that any cell loaded with stored lipoid material would fail to

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be stained at all strongly by intravital dyes; doubt has been cast on this b Schuleman (1917), who demonstrated that macrophages laden with vit dyes were still stainable in the normal manner.

Sheldon (1935) was of the opinion that the interstitial cells were rarel involved in haemochromatosis in man, so supporting the investigations c Polson (1929) in the rabbit. On the other hand, Nissim (1953 *a*, *b*; 1955 using massive doses of either ferric chloride caramelate or an iron-dextra complex ('Imferon', Benger Laboratories) in mice, found that Leydig cell took up these substances.

The divergence of opinion as to whether cells can exhibit an endocrine a well as a 'phagocytic' function is also found in relation to other ductles glands, notably the ovary (Borell, 1919; Cappell, 1929; Bratianu, 1930*b*).

Materials and Methods

Twenty healthy, sexually-mature, albino rats were used in this inves tigation. Two intravital dyes were employed; trypan blue and Unna' polychrome methylene blue. These were used either singly or in variou combinations (table 1).

The medium used was a 0·2% solution in the dye, sterile distilled wate being used as the diluting fluid. The standard dose was 1·5 ml of the solutior per 50 g of body-weight; this was given as a single intraperitoneal injection

In two of the experimental animals the amount of solution given was much greater, 30 ml and 150 ml respectively being injected as a single dose in th former, and in 5 equally divided doses on alternate days in the latter case Autopsy on the rat which had received the larger amount of trypan blu revealed that some 5 ml of dark blue fluid remained in the peritoneal cavity no evidence of peritonitis being discovered. It was found that if a scanty distribution of trypan blue granules was required, in order to obtain a clea picture of the cellular structure, the best results were obtained by halving th standard dose.

The animals were usually not killed before the 7th day following the las injection of dye, because the granules of dye were found to be much mor clearly defined at this than at earlier periods.

It will be seen from table 1 that several additional procedures were als carried out:

(*a*) unilateral interruption of the testicular artery at a point before its anastomosis with the vasal artery.

(*b*) unilateral interruption of the testicular artery, with removal of the con- tralateral testis, as described by Harrison (1953). This was performed 7 days after the injection of the standard amount of dye.

(*c*) 2·5 mg of testosterone propionate were given by intraperitoneal injection on alternate days for the periods of time indicated.

(*d*) unilateral orchidectomy was carried out, with aseptic precautions, on 2 rats, 7 days after they had been injected with the standard amount of dye.

(*e*) laparotomy was performed on 2 rats and, after the testes had been

elivered into the wound, 0·5 ml of trypan blue solution were injected underneath the tunica albuginea of each testis.

The figures given in the right-hand column of table 1 indicate either the umber of days' survival after injection or, when a further procedure has een carried out, the survival period after this.

It was noted that the above-mentioned intravital dyes were non-toxic and d not produce sensitization when given in the amounts and in the dilution dicated in these experiments.

TABLE 1

umber f rats	*Dye used*	*Amount of dye*	*Operation performed or drug used*	*Days elapsing between experimental procedure and killing of animal*
2	Polychrome methylene blue (Unna)	S.D.	..	7
3	Trypan blue	S.D.	..	4, 7, 60
1	Trypan blue	4 ml	..	7
1	Trypan blue	30 ml	..	7
1	Trypan blue	150 ml	..	7
2	Trypan blue	S.D.	Testosterone propionate 2·5 mg on alternate days	20, 35
4	Trypan blue	S.D.	Interruption of testicular artery	1, 5, 10, 15
2	Trypan blue	S.D.	Interruption of testicular artery and unilateral orchidectomy	7, 11
2	Trypan blue	0·5 ml	Injection of dye direct into testis	7
2	Trypan blue	S.D.	Unilateral orchidectomy (left)	7, 35

S.D. = standard dose = 1·5 ml per 50 g of body-weight.

Trypan blue was most frequently employed as this dye was found to give e most satisfactory intravital staining during the preliminary tests.

On removal from the body the testis was placed in Heidenhain's 'Susa' ative for 1 hour, after which time a segment was removed from the equator the testis with a sharp razor and replaced in the fixative. Five-micron ctions, parallel to the equator of the testis, were lightly counterstained h Heidenhain's iron haematoxylin and eosin (Gurr, 1953). In the case of testis of the rats subjected to unilateral orchidectomy, serial sectioning in 25) of the whole organ was carried out.

The seminal vesicles with the coagulating glands and a narrow bridge of ostatic tissue were removed at autopsy, and weighed, after having been dried r calcium chloride for 48 h.

Results

Trypan blue stains the following four types of cell:

cells which are morphologically indistinguishable from the interstitial cel of Leydig (fig. 1, G); certain of the fibroblasts, the majority of these bei applied to the outer surface of the basement membrane of the seminifero tubule (fig. 1, C); certain of the perivascular cells (fig. 1, B); small cells wi irregular outlines, about 10 to 20 μ in diameter, the structure of which obscured by the nature of the staining with the intravital dye. In the pr ceding three types of cell the dye is aggregated into well-defined granule whereas in this type of cell, the granules are more widely separated and sho a lack of definition (fig. 1, A).

Variations in the amount of dye injected do not produce any difference either the number or type of intertubular cells which are stained by the dy

The distribution of mast cells in the testis was investigated by the metho described above. They are found to be present within the tunica albugin (fig. 1, E), immediately underlying this structure (fig. 1, F), and in the co nective tissue of the epididymis (fig. 1, D), but are absent from the intertubul tissue.

Under normal conditions cells containing dye are not found within th basement membrane of the seminiferous tubules. After the production ischaemia of the testis by ligation of the testicular artery, there is a mark passage of these cells from the intertubular tissue into the seminifero tubules. During the first 48 h after operation some of these cells lie close to th inner surface of the basement membrane of the tubules, whilst others a situated either among the actively dividing spermatogonia, or on the surfa of the desquamated mass of tubular cells (fig. 1, G). By the 3rd day they a widely scattered throughout the tubular contents (fig. 1, H), and the intr cellular dye, with the exception of that contained within the cells in the sub epididymal region of the testis, has lost its granular form, staining both th cytoplasm and nucleus in a diffuse manner.

The injection of trypan blue directly under the tunica albuginea of th testis results in similar histological findings to those described above, exce

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Fig. 1 (plate). A, interstitial cell showing lack of definition of intravital dye granules an irregularity of cellular outline. (Trypan blue; counterstained with haematoxylin and eosin.)

B, section of blood-vessel demonstrating presence of dye granules in one perivascular ce (Same technique as A.)

C, two peritubular fibroblasts, only one of which contains dye. (Same technique as A.)

D, mast cells in connective tissue of the epididymis. (Unna's polychrome methylene blu

E, mast cells in tunical albuginea of the testis. (Same technique as D.)

F, mast cells underlying the tunica albuginea of the testis. (Same technique as D.)

G, two interstitial cells, only one of which contains granules of intravital dye. (Trypan blu azo-carmine counterstain.)

H, cells containing dye are widely scattered among contents of a seminiferous tubule. (Sam technique as A.)

I, cells containing dye are seen among the spermatogonia and on surface of desquamat mass of tubular cells. (Same technique as A.)

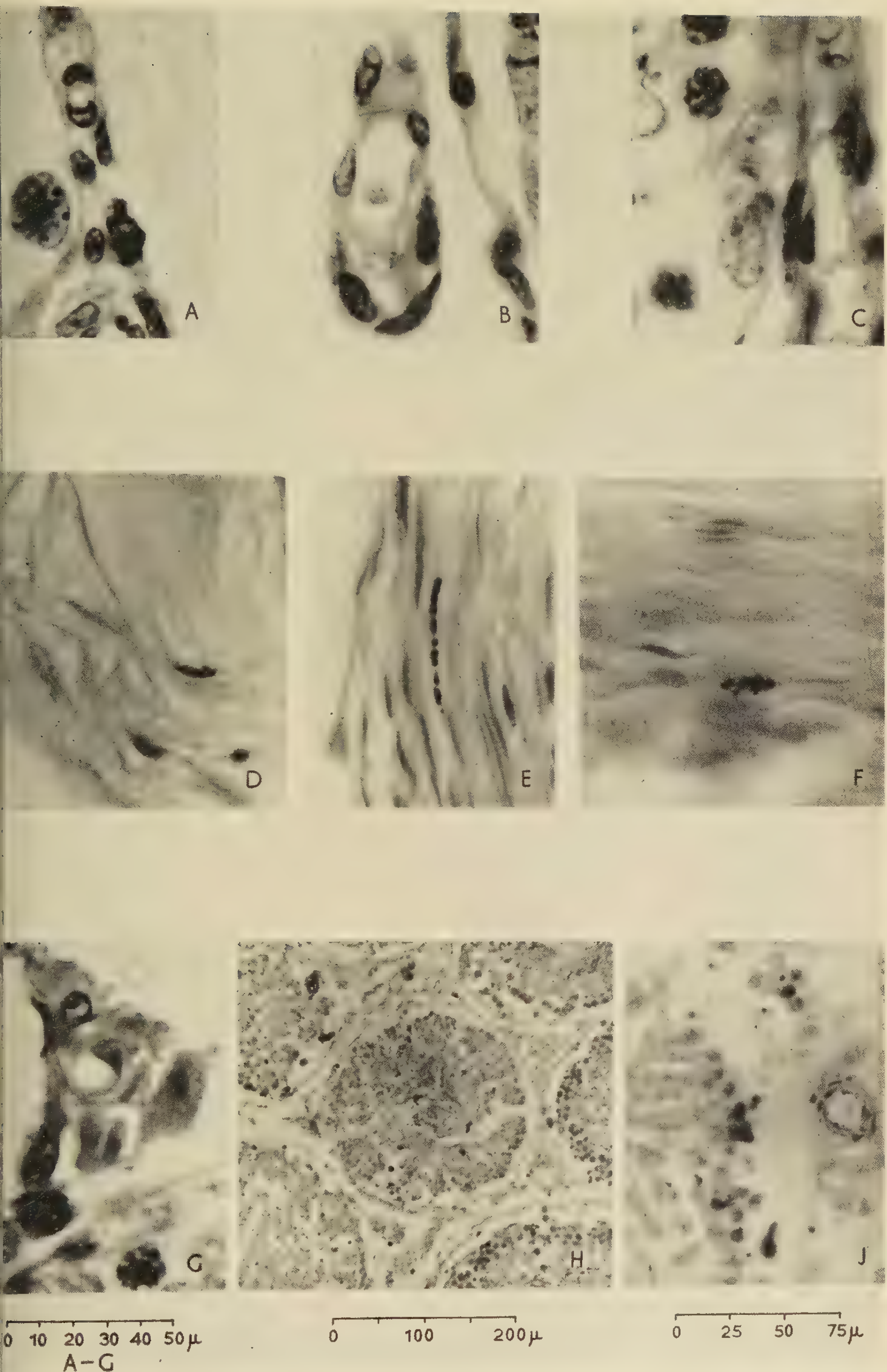

Fig. 1

J. H. GRANT

at the dye is confined to a circumscribed area of this organ, and that all the lls in this area contain massive quantities of dye.

The administration of testosterone propionate leads to a gradual diminuon in the number of interstitial cells not intravitally stained, and to an crease in the amount of intertubular fluid. It has little or no effect on the travitally stained Leydig cells. No significant difference is noted between e weights of the seminal vesicles of the animals which have been injected ith dye and those of the control series. In the former group of rats, signs castration are invariably absent.

Discussion

It is clear from the results of these experiments that interstitial cells are ivisible into a group consisting of those which are stained by trypan blue eticulo-endothelial interstitial cells) and a group which are not (true Leydig ells). By definition the former belong to the reticulo-endothelial system as rst described by Aschoff. The mechanism by means of which colloidal ital dyes gain access into these cells is uncertain, but it has been suggested hat 'ultra phagocytosis' or 'colloidoplexy' are responsible (Robertis, owinski, and Saez, 1950), whatever these terms may imply. Soluble dyes ke methylene blue or neutral red, which are not colloidal but which neverheless, like trypan blue, stain cells intravitally, manifestly cannot be taken in y phagocytosis or colloidoplexy.

The occurrence of these two morphologically identical but functionally ifferent types of cell corresponds to the two varieties of benign interstitial ell-tumour. Although it has been stated that the reason why these neoplasms o not usually give rise to manifestations in post-puberal patients is because e secondary sexual characteristics are already established, this may equally ell be due to the occurrence of reticulo-endothelial interstitial cells in these ases. As Leydig cell-tumours are associated with precocious puberty in hildren (Stewart, Bell, and Roehlke, 1936), it would appear that pre- and ost-puberal benign interstitial cell neoplasms arise from true and reticulondothelial Leydig cells respectively.

Maximow (1899) describes numerous large Leydig cells undergoing mitotic ivision around the periphery of experimentally produced testicular injuries. hese cells are, without doubt, the reticulo-endothelial type of interstitial ell, which are also probably the ones which Testa (1929) refers to when he escribes interstitial cells as playing an active role in the formation of granulaon tissue. It is suggested that if one postulates that the reticulo-endothelial nd true Leydig cells multiply by mitotic and amitotic division respectively, e discrepancy between the work of Maximow (1899) and Testa (1929), and hat of Barbeleben (1897), Ancel and Bouin (1903), Stieve (1922), and Stein 931) is explicable on the grounds that the two first-mentioned authors ased their observations on material in which the reticulo-endothelial interitial cells predominate.

It is suggested that the difference between the results obtained in the present series and those reported by Nissim (1955) is due to the use of iron-dextran complex Imferon, which in the amounts used would lead to the formation of widespread fibrous tissue. Goldberg, Fee, and Martin (1955) point out that the amounts of Imferon used by Nissim (1955) produce a metallic iron concentration of 500 mg per kg of body-weight in the mouse, whereas in man the amount of iron is unlikely to exceed 50 and is usually about 20 mg per kg of body-weight. This, it is submitted, in long-term experiments, would result in the obliteration of the greater number of the interstitial cells by fibrous tissue, and give rise to an appearance which suggests an uptake of the iron-dextran complex by the majority of the intertubular cells.

The presence of four morphologically-distinct cell types in the testis, all of which are stained by intravital dyes, as opposed to the majority of other organs which contain only two, may be explained by the transition of one cell type into another (Ranvier, 1890, 1900; Möllendorff and Möllendorff, 1926; Esaki, 1928).

The passage of reticulo-endothelial interstitial cells from the intertubular tissue to the seminiferous tubules after the production of testicular ischaemia is most readily explained by the assumption that in the ischaemic testis the latter are the only routes by which these cells can be removed. In support of this it has been shown by Grant (1955) that cellular debris from the seminiferous tubules can be seen in the ductus epididymidis under these circumstances, and that the decrease in testicular volume following interruption of the testicular artery is so rapid that it is explicable only in this way. A second possible explanation is that there is a greater liberation of 'leucotaxine' (Menkin, 1938) from the cells of the seminiferous tubules, than from those of the intertubular tissue. Both these processes may operate at one and the same time.

The nature of the small cells with the crenated outlines is difficult to determine because, as has been previously mentioned, their morphological features are frequently obscured by dye. The diffusion of the granules of dye implies that these cells are undergoing degenerative changes, the irregular outline of the cytoplasm confirming this. These characteristics indicate that the cells correspond to the decrepit type of interstitial cell (Regaud, 1900).

The distribution of mast cells described above is of importance, since Duthie and Barker (1955) suggest that these cells may have a function in the reaction to trauma; their absence from the greater part of the testis may explain the poor regenerative powers exhibited by this organ.

My grateful thanks are due to Professor R. G. Harrison for his constant help and advice. I also wish to thank Mr. L. G. Cooper and Miss B. Birkett for their technical assistance, as well as Dr. W. J. Tindall and Organon Laboratories Limited for providing the testosterone propionate used in this investigation.

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A Histochemical Study of Dehydrogenase Activity in the Pectoralis Major Muscle of the Pigeon and certain other Vertebrate Skeletal Muscles

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SUMMARY

Certain dehydrogenases in the breast muscle of the pigeon and fowl and the leg iscle of the fowl and frog were studied histochemically by the use of 2:3:5:triphenyl razolium chloride. The dehydrogenase activity was found to have a relationship th the colour and the mitochondrial content of the individual muscle fibres. In the geon breast muscle, however, the broad white fibres did not show the presence of y of the enzymes studied. It is therefore concluded that these fibres in the pigeon east muscle are a unique system in which none of the oxidative processes concerned kes place; they cannot be considered as analogous to the white fibres of the other iscles studied.

INTRODUCTION

SINCE George and Jyoti (1957) showed that fat is the chief fuel during sustained activity of the breast muscle of flying birds and George and aik (1958*a*) that the white and red fibres in the pectoralis major muscle of the geon are respectively loaded with glycogen and fat, this muscle has become e subject of more extensive studies in our laboratories in the hope of dis-vering the functional significance of these distinct types of fibres existing le by side in one and the same system. George and Naik (1958*b*) studied the lative distribution of mitochondria in the two types of fibres and observed at the mitochondria occur in large numbers in the narrow red fibres while e broad white ones contain few or none. George and Scaria (1956) showed at the pectoralis major muscle of the pigeon contains a lipase and presented idence to show that the lipase is confined only to the narrow fibres (1958). ecently George, Nair, and Scaria (1958) studied the alkaline phosphatase tivity in this muscle by histochemical methods and found that the zyme is located mostly in the narrow fibres and only at the border in the oad fibres. They suggested that most of the transphorylation reactions in e muscle takes place in the narrow fibres and the presence of alkaline iosphatase at the periphery of the broad fibres may be for the transport of ycogen from the broad fibres to the narrow ones. Other workers have recently udied the dehydrogenase activity in the pigeon breast muscle by chemical ethods (Chappel and Perry, 1953). In this paper we report the result of a udy undertaken to demonstrate the dehydrogenase activity in the pectoralis ajor muscle of the pigeon (*Columba livia*) by histochemical methods. This udy was further extended to the gastrocnemius and the pectoralis major uscles of the fowl (*Gallus domestica*) and the former muscle of the frog (*Rana*

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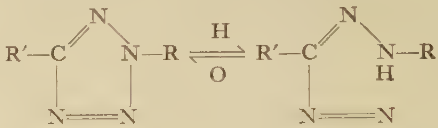
tigrina) for comparison. The following dehydrogenases were studied: succini malic, lactic, D-glucose, and glycerophosphate dehydrogenase.

Method

Several authors have used the TTC (2:3:5:triphenyl tetrazolium chlorid method for the study of succinic dehydrogenase in animal tissues (Straus an others, 1948; Black and Kleiner, 1949; Seligman and others, 1949; Bla and others, 1950). We followed the method of Straus and others (1948) an Pearse (1954) for succinic dehydrogenase and extended its application to th other dehydrogenases. The principle of the method is that the colourle soluble tetrazolium salt on reduction is converted to an insoluble re compound, formazan, which is deposited at the sites of reduction in th tissues. The reduction is brought about by the enzymic liberation of hydroge from the substrate. The tetrazolium salt acts as the hydrogen acceptor. Th reaction is as follows (Pearse and Scarpelli, 1958).



Tetrazolium *Formazan*

In biological systems this reaction is not reversible, and the quantity formazan deposited in tissue sections can be directly related to the amount succinic dehydrogenase (Defendi and Pearson, 1955). In the case of all th above enzymes except succinic dehydrogenase the tetrazolium salt cannot a as the hydrogen acceptor without the intervention of a cofactor (DPN) whic functions as a hydrogen carrier. Reduction of the TTC by reducing sub stances such as glutathione, cysteine, ascorbic acid, or reducing sugars usuall present in tissues does not take place under the conditions of our experimen (Pearse, 1954). The incubation medium in each case contained 1·5 ml 0·1 M phosphate buffer of pH 7·2, 1 ml 0·1 M solution of the substrate, 7·5 mg TTC, and 0·625 mg of DPN in a total volume of 2·5 ml in a cuvette. DP was omitted from the mixture for succinic dehydrogenase, since this enzym does not require the cofactor (Baldwin, 1953). Sections about 50 to 80 μ thic prepared according to the method of George and Scaria (1958), were immerse in the respective incubation media, covered with a lid, and incubated for 5 t 30 min at 37° C. They were then washed in buffer, fixed in 10% neutr formalin, washed in water, and mounted in glycerine jelly without counte staining.

Shelton and Schneider (Pearse, 1954) claimed that freezing destroys endo genous activity of succinic dehydrogenase. We have observed that froze sections prepared as cited above do show endogenous activity when directl transferred to TTC solution; thus freezing alone does not destroy endogenou activity. But the endogenous activity was found to be lost when the section were kept for 10 to 15 min in cold 0·1 M phosphate buffer at pH 7·2 befor

nsferring them to the TTC solution. So in this study all the sections were ced in phosphate buffer for 10 to 15 min to ensure complete loss of endo-ous activity, and then transferred to the respective incubation media. posure of the incubation medium to bright sunlight was recommended arse, 1954) for hastening the reaction and reducing the period of incuba-n. We have noticed that this procedure might produce erroneous results ce colour development under such conditions is not due to enzymic activity ne, because the solution itself turns red on exposure to sunlight.

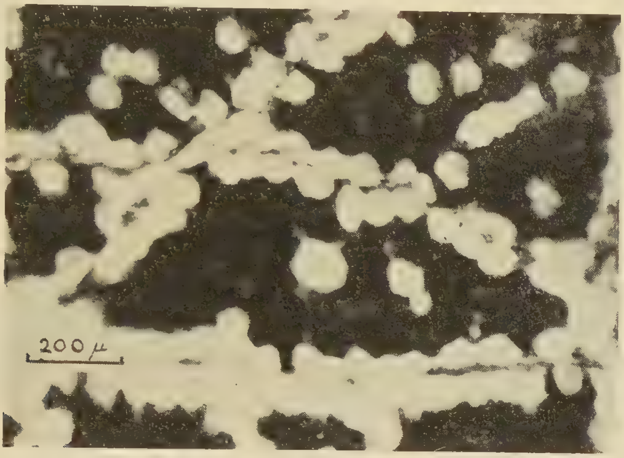


FIG. 1. Photomicrograph of a transverse section of the pectoralis major muscle of the pigeon, showing localization of succinic dehydrogenase in the narrow fibres. The broad fibres stand out as clear, colourless areas.

RESULTS AND DISCUSSION

It was found that the pigeon breast muscle contains all the dehydrogenases cept D-glucose dehydrogenase. Colour development in the sections was oid enough to be visible within 5 to 10 min of incubation. Microscopic amination of the sections revealed that the enzymic activity is confined only the narrow fibres as was indicated by the deep-red colour developed in em. The colour development was so intense as to obscure the boundary of individual narrow fibres (fig. 1). Short periods of incubation clearly owed the mitochondria as deep-red spots in the narrow fibres. The broad res were completely blank and stood out from the rest of the fasciculus clear, colourless areas. It could, therefore, be concluded that the broad fibres the pigeon breast muscle do not contain any dehydrogenase; or, if they tain any, only extremely minute traces, which we could not detect. This clusion is further substantiated by the fact that when sublethal doses of C were administered to the pigeon intravenously or intramuscularly and bird was killed after a day or two, formazan could be detected at the centres highest metabolic activity such as liver, kidney, adipose tissue, and the

heart and breast muscles. In breast muscle, the colour due to formazan cou[ld]
be noticed only in the narrow fibres.

From our observations on other muscles also, a correlation could be draw[n]
between the dehydrogenase activity, the colour of the muscle, and the
mitochondrial content (table 1). A similar relationship was shown by Pa[ul]
and Sperling (1952), who used other methods.

TABLE 1

Dehydrogenase activity in various muscles

Animal	*Muscle*	*Colour*	*Abundance of mitochondria*	*Time taken for maximum colour development*	*Intensity of th[e] colour*
Frog	gastrocnemius	white	none	++++	+
Fowl	pectoralis major	white	very few	+++	++
Fowl	gastrocnemius	pale red	more than in pectoralis major of fowl	++	+++
Pigeon	pectoralis major	red	more than in any of the other muscles	+	++++

The abundance of mitochondria was determined by microscopic examination of the stain[ed] sections.

+, minimum; ++++, maximum.

In the leg muscle of the fowl, where different types of fibres varying fro[m] red to white with all intermediate forms occur, dehydrogenase activity w[as] detected in all the fibres. But the mitochondrial content and the dehydro[-]genase activity was found to vary, the maximum and the minimum being [in] the red and the white fibres respectively. In the pigeon, on the other han[d,] the white fibres in the breast muscle did not show any indication of th[e] presence of any of the enzymes for which tests were made. We are therefo[re] led to believe that none of the oxidative processes concerned takes place [in] the broad fibres of the pigeon breast muscle. If this conclusion is correct, th[e] broad white fibres of the pectoralis major muscle of the pigeon should b[e] considered as unique and not as analogous to the white fibres of any of th[e] other muscles studied. Further investigations which are in progress will, w[e] hope, throw more light on the physiology of these unique fibres in the pigeo[n] breast muscle and perhaps on the general problem of muscular contraction [as] well.

We are grateful to the Sigma Chemical Company, U.S.A., for their generou[s] gift of the TTC and DPN used in this study.

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Histochemical and Morphological Studies of Lipids in the Oogenesis of *Pheretima posthuma*

By VISHWA NATH, BRIJ L. GUPTA, *and* S. L. MANOCHA

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With one plate (fig. 2)

Summary

A study of the oocytes of the earthworm, *Pheretima posthuma*, examined fresh under the phase-contrast and interference microscopes as well as by histochemical techniques, has revealed that there are two types of lipid bodies in the cytoplasm. The lipid bodies of the first type (L_1) are smaller, appear as homogeneous, dark granules under the phase-contrast microscope, and have a protein-phospholipid core surrounded by a thick sheath of phospholipids only. The lipid bodies of the second category (L_2), which arise as a result of growth and chemical change in L_1 bodies, have a pure phospholipid core surrounded by a thick triglyceride sheath. They give a ringed appearance under the phase-contrast microscope. The study under the interference microscope shows that this ringed appearance is an optical artifact. The lipid spheres present in the follicular epithelium contain phospholipids only.

The mitochondria are in the form of minute granules. They remain unchanged throughout oogenesis.

Some vacuoles devoid of any lipids, proteins, or carbohydrates have been observed. They also remain unchanged.

Pure triglyceride spheres, yolk globules, nucleolar extrusions, as well as cholesterols and cholesteryl esters are absent.

Introduction

THE inadequacy of the osmium and silver techniques for the so-called Golgi apparatus has been brought out by the confusion that still exists with regard to our knowledge of the chemistry of the cytoplasmic inclusions of the earthworm egg, in spite of at least a dozen papers previously published on the subject (see Nath, 1957).

In our present study of the cytoplasmic inclusions of the egg of the earthworm, *Pheretima posthuma*, we have employed phase-contrast and interference microscopy on living material, and histochemical techniques. Our studies have once more emphasized that the so-called Golgi techniques of osmium and silver are inadequate, indeed misleading, in studies of the chemistry and morphology of cytoplasmic inclusions.

Material and Technique

The specimens of *P. posthuma* used in the present study were collected during the months of July and August, 1956 and 1957. Material was also collected during the remaining months of the year for study of seasonal variations in the ovaries. The animals were dissected alive in the physiological solution of Baker (1944), i.e., 0·2 ml of 10% anhydrous $CaCl_2$ in 100 ml of

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0·7% NaCl solution, and the ovaries were transferred directly to the vario
fixing fluids.

The fixative employed for almost all the histochemical tests was formald hyde-calcium followed by postchroming, as recommended by Baker (194 for his acid-haematein technique. The ovary was embedded in gelatine for t cutting of frozen sections. A complete list of the histochemical reactions us for the detection of lipids, proteins, and carbohydrates is given in table on page 477.

The action of lipid-solvents was also investigated. For details of the metho used, see Nath and others (1958). Fresh ovarioles were in some cas exposed to the solvents. In other cases ovaries were fixed in formaldehyd calcium, and sometimes postchromed; gelatine sections were prepared, a the solvents acted upon these. Hot solvents could not be used as it is impossib to manipulate the ovaries on account of their very small size, even in a micr Soxhlet extractor.

Most of the histochemical reactions listed in table 1 were also employ on centrifuged material. The ovaries for this purpose were centrifuged for o hour in Baker's physiological solution. A Christ (W. Germany) electr centrifuge was used at 5,000 rev/min. The ovaries were subsequently fix by the usual formaldehyde-calcium/postchroming technique. The stratific tion achieved was excellent.

Living ovaries were studied by phase-contrast and interference microsco The ovaries were mounted in a drop of Baker's physiological solution, witho teasing. The pressure of a no. 1 coverslip was sufficient to flatten the oocyt satisfactorily. The margins of the coverslip were sealed with vaseline to avo evaporation. A Zeiss 'W' microscope with Ph 100/1·25 objective and K eyepiece was used for phase-contrast studies. A Zeiss micro-reflex attachme with Contax camera was used for photomicrography. Kodak panchroma 35-mm film was used. A Charles Baker instrument with both shearing a double-focus objectives was used for interference microscopy.

The term 'lipid' is used in this paper to mean triglycerides and all oth cellular constituents having similar solubilities (phospholipids, etc.).

Results

Morphological observations

The germ-cells in the ovary of *P. posthuma* are arranged in the form filaments, radiating from the point of septal insertion of the ovary. U differentiated germ-cells and oogonia are present at the proximal end of ea filament (ovariole), whereas the oldest oocyte occupies the distal end. A the developmental stages of an oocyte are arranged serially in a sing ovariole; this greatly facilitates the study of oogenesis.

The use of the various physical lipid colorants has revealed that the lipi are present in the form of homogeneous, spherical bodies in the cytoplas during all stages of oogenesis. The number and size of these lipid spher increase considerably with the growth of the oocyte. In fact, the wh

TABLE I. *Table showing the histochemical reactions of the oocytes of* Pheretima posthuma

Technique	*Fixative*	*Embedding medium*	*Thickness of sections* (μ)	*Reference*	*Mito-chondria*	*Bodies in the follicular epithelium*	L_1	L_2	*Vacuoles*
Sudan black B:									
in 70% ethanol	FS+PC	G	10	Baker, 1949, 1956	++	N	+++	A	−(P)
	FCa+PC				++	++	+++	+++ (Some ringed)	,,
in 70% ethanol at 60° C	,,	G	10	Chiffelle and Putt, 1951	++	++	+++	+++	,,
in propylene glycol at 60° C	,,	G	10	Krishna, 1950	++	++	+++	+++	,,
— after treatment with cold acetone	Fr. and FCa	G	10	Pearse, 1954	++	++	+++	++(R)	,,
— — cold ethanol	,,	G	10	,,	−(P)	O	−(R)	O	,,
— — cold ether	,,	G	10	,,	−(P)	O	−(R)	O	,,
— — methanol/chloroform	,,	G	10	,,	−(P)	O	−(R)	O	,,
Sudan III and IV:									
in 70% ethanol/acetone	FCa+PC	G	10	Kay and Whitehead, 1941	+	++	++	+++	
in 1% gelatine suspension	,,	G	10	Govan, 1944	++	++	++	+++	,,
Fettrot in 70% ethanol	,,	G	10	Pearse, 1954	+	++	++	+++	,,
Nile blue	,,	G	10	Cain, 1947, 1948	blue	blue	blue	violet	,,
Acid haematein (AH)	,,	G	10	Baker, 1946	++	+++	+++	++(R)	,,
— after pyridine (PE)	WB+PE	G	10	,,	−	O	++(R)	O	,,
Fischler's reaction	FS	G	10	Pearse, 1954	−	−	−	−	,,
Periodic acid / Schiff (PAS)	FCa+PC	G	10	Hotchkiss, 1948 Pearse, 1954	−	−	−	−	,,
,, ,,	Carnoy	P	$7\frac{1}{2}$	,,	−	−	−	−	,,
Mercuric bromphenol blue (Hg–BPB)	FCa+PC	G	10	Mazia and others, 1953	++	−	+(R)	−	,,
,, ,,	Carnoy	P	$7\frac{1}{2}$	,,	++	−	+(R)	−	,,
— after treatment with ethanol	Fr. and FCa	G	10	,,	+++	−	++(R)	−	,,
— — pyridine	WB+PE	G	10	,,	+++	−	++(R)	−	,,
Performic acid / Schiff (PFAS)	FCa+PC	G	10	Pearse, 1951	−	+++	−	+++	,,
— after bromination	,,	G	10	Lillie, 1952	−	−	−	−	,,
Plasmal reaction	,,	G	10	Hayes, 1949 Cain, 1949	−	−	−	−	,,
Cholesterol reactions	,,	G	10	Schultz, 1924 Gomori, 1952 Romieu, 1927 Pearse, 1954	−	−	−	−	,,

Key: A = Absent; FCa = formaldehyde-calcium; FS = formaldehyde-saline; Fr. = fresh material; G = gelatine; N = not observed; P = paraffin; −(P) = present but negative; PC = with postchroming; (R) = reduced in size; WB+PE = weak Bouin's fluid followed by pyridine extraction; + = weak reaction; ++ = moderate reaction; +++ = strong reaction; — = negative; O = dissolved.

process of oogenesis in earthworms, so far as the cytoplasm is concerned, restricted to the synthesis and growth of the lipid spheres. There is no indica tion of the presence of any protein or carbohydrate yolk at any stage oogenesis.

The cytoplasm of the earliest cell studied in an ovariole reveals the presen of four or five minute lipid granules (fig. 1, A, B). With the growth of the oocy the number of the lipid granules increases and the cytoplasm now also shov a mass of mitochondria near the nucleus (fig. 1, C). Gradually the mitochondr and the lipid spheres become uniformly scattered throughout the cytoplas of the developing oocyte (fig. 1, D–G). The mitochondria do not change at even in the oldest oocyte studied (fig. 1, H, I). The size of the largest lip sphere in the oldest oocyte studied is approximately 3·5 μ (fig. 1, I).

Even in the youngest oocyte studied, the cytoplasm reveals the presence some vacuoles in all the preparations. The number of these vacuoles increas considerably, and some of them invade the central regions of the cytoplas also. These vacuoles persist even in the oldest oocyte without any visib change (fig. 1, A–I).

The nucleus remains small throughout oogenesis, and is almost invariab excentric. It always contains a single nucleolus.

In very young oocytes the small lipid globules appear by phase-contra or interference microscopy as homogeneous bodies giving a high phas change. One or two larger spheres that are present at this stage show a ring appearance: a grey medulla is seen to be surrounded by a very thick, dar cortical sheath when positive phase-contrast microscopy is used (fig. 2, A, B The histochemical tests described in the later part of this paper have show that these larger bodies are chemically different from the smaller ones. Th have been called L_2 bodies, while the smaller ones, which appear homogeneo under phase-contrast, are called L_1. There is a gradual increase in the numb and size of the L_2 bodies, which continue to give a ringed appearance und the phase-contrast microscope (fig. 2, C, D). The mitochondria always appe as minute grey granules under the phase-contrast, whereas the vacuoles com pletely escape detection, probably because the cytoplasm is choked with th lipid bodies. Moreover, the out-of-focus images of the lipid bodies also appe as vacuoles; thus it is not easy to differentiate them from the real vacuole

When oocytes are studied under the interference microscope and th analyser is so set as to give a pinkish-yellow coloration to the cytoplasm, th larger spheres (L_2) show a deep violet cortex surrounding a blue medull A general diffused, though patchy, violet coloration is also present througho the cytoplasm; this might be due to the presence of mitochondria. Som vacuoles are also visible. If a blue-green filter is now used in front of the lig source, the picture presented by these spheres is quite similar to that und the phase-contrast microscope. But when the analyser of the interferen microscope is so set that the colour of the cytoplasm is yellow, the colour both the L_1 and L_2 spheres is pinkish violet, and the L_2 spheres do n exhibit a duplex or ringed structure at all.

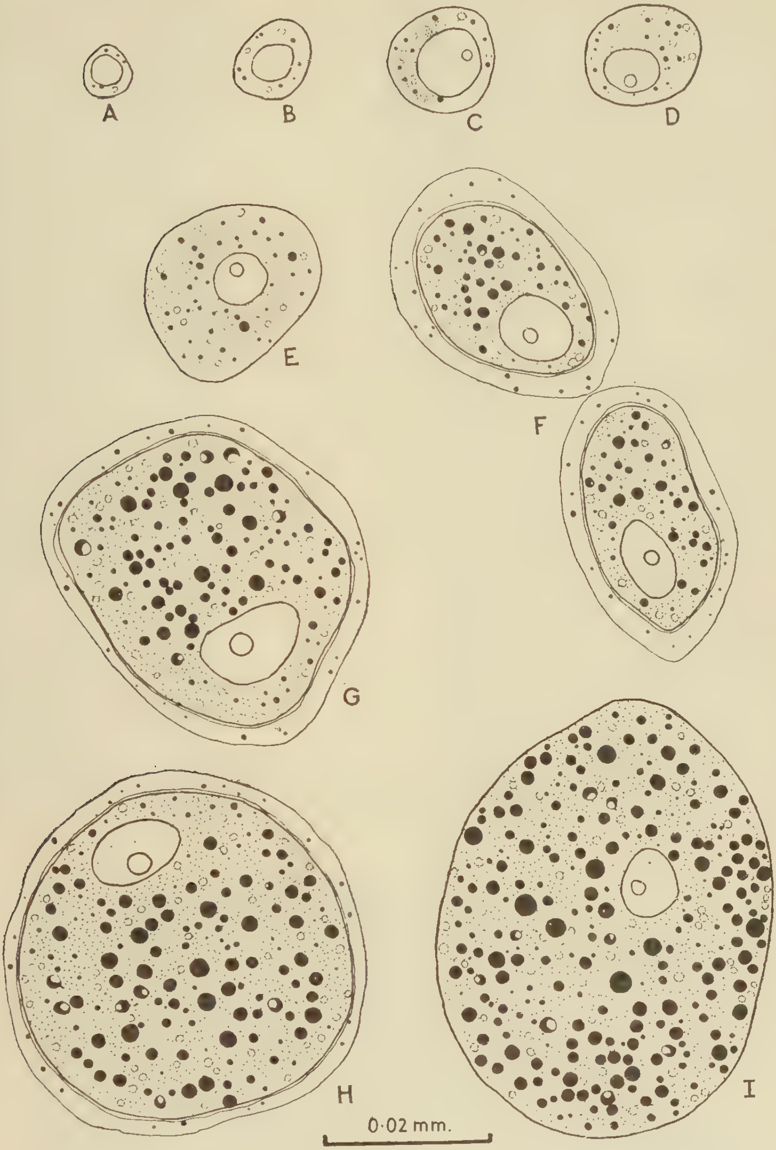

FIG. 1. Camera lucida drawings of the oocytes of *P. posthuma*, showing the distribution of the lipid bodies, vacuoles, and mitochondria during oogenesis, and their reaction to Sudan black B after fixation in formaldehyde-calcium and postchroming. A and B, very young oocytes showing a few lipid bodies and vacuoles but no mitochondria. C and D, oocytes showing the appearance and gradual dispersal of the mitochondria. E–H, oocytes showing the growth of the cell and increase in the number of lipid bodies, mitochondria, and vacuoles. I, largest oocyte studied. Note the increase in the size of the lipid bodies, some of which (L_2) have a ringed or crescentic appearance.

The above observations suggest that the duplex or ringed appearance of the L_2 spheres under the phase-contrast and interference microscopes is an optical artifact produced by a great difference in the phase displacement of light caused by the lipid bodies in comparison with the ground cytoplasm. This difference, when reduced by changing the analyser of the interference microscope and thus reducing the over-all contrast, results in the disappearance of the duplex spheres, which now appear homogeneous.

The homogeneous nature of the lipid bodies is stressed in the histochemical section of this paper.

Centrifuged oocytes reveal the following stratification under the phase-contrast microscope, as well as by lipid tests (figs. 2, E, F; 3, C).

(i) The vacuoles go to the centrifugal pole and generally collapse.

(ii) The mitochondria as well as the small lipid bodies (L_1) form a distinct stratum, next to the heavy, vacuolar substance.

(iii) The third stratum is formed by the hyaloplasm containing the nucleus with its nucleolus.

(iv) The centripetal pole is occupied by the larger lipid spheres (L_2), which give the usual ringed appearance under the phase-contrast microscope (fig. 2, F).

Histochemical reactions

The various histochemical reactions tried and their results are summarized in table 1 on page 477.

Mitochondria. The mitochondria have the usual phospholipid-protein nature which remains unchanged throughout the course of oogenesis.

The PFAS for unsaturated bonds (HC=CH) as given by Lillie (1952) and Pearse (1951) invariably gave a negative reaction with the mitochondria, although natural phospholipids are usually highly unsaturated. The very small size of these granules may be the cause of this failure.

Lipid bodies. Some of the L_2 bodies are not homogeneously coloured by Sudan colouring agents but appear as crescents or rings. This is due to the presence of some lipids which are solid at room temperature (12° C to 40° C). Almost all of them lose their crescentic appearance when coloured with Sudan black B in propylene glycol at 60° C. In this respect they are comparable to some of the L_3 bodies described in cockroach by Nath and others (1958).

FIG. 2 (plate). Photomicrographs of the living oocytes of *P. posthuma*, taken by positive phase-contrast microscopy.

A, a very young oocyte showing the optically homogeneous L_1 bodies in the cytoplasm.

B–D, oocytes showing a gradual increase in the size of the cell as well as in number and size of the lipid bodies; the larger lipid bodies (L_2) have a ringed appearance.

E, a large centrifuged oocyte, showing four distinct strata of the cell inclusions (further explanation in text).

F, an oocyte which is only partially centrifuged. Note the L_2 bodies, which appear as distinct rings; they have moved towards the centripetal pole. The heavy substance has just started accumulating at the centrifugal pole.

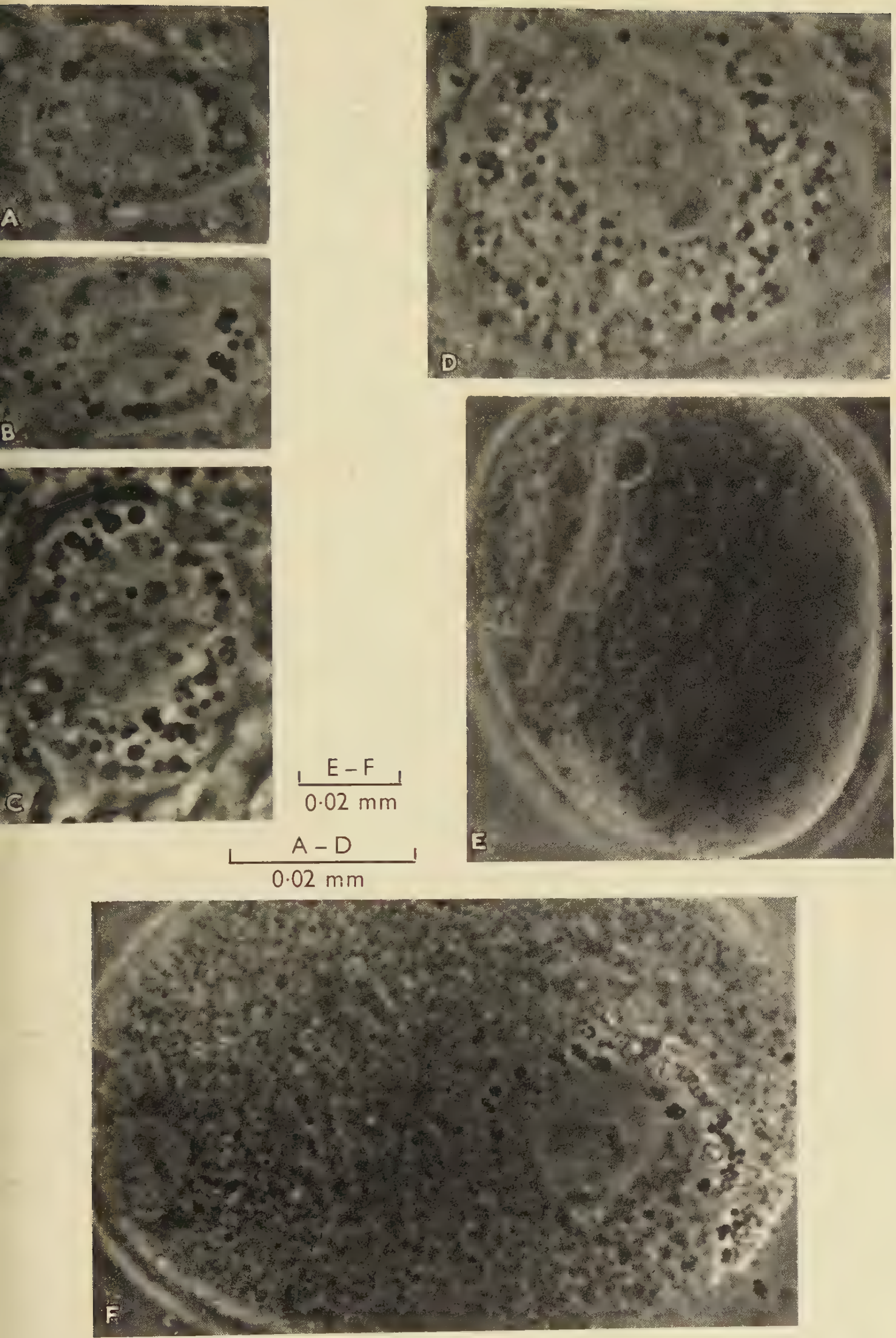

FIG. 2

V. NATH, B. L. GUPTA, *and* S. L. MANOCHA

The results of the acid-haematein (AH) test of Baker (1946) with its pyri-
ine extraction (PE) control require special mention. The L_1 bodies give a
niform, intense, blue-black coloration in the AH test; this indicates the
resence of phospholipids and/or phosphatidic acids (Casselman and George,
952). The absence of the latter is shown by the negative reaction which both

G. 3. Camera lucida drawings of the oocytes of *P. posthuma*. A and B, a young and an old cyte from an acid-haematein preparation. Note the reduction in the size of L_2 bodies in B ompare fig. 1, H), which appear almost as small as L_1 bodies. The single nucleolus is also I-positive. C, an oocyte from a centrifuged ovary fixed in formaldehyde-calcium, post-romed, the sections coloured subsequently with Sudan black B. Note four distinct strata. (Further explanation in text.)

e L_1 and L_2 bodies give to Fischler's test for fatty acids (Pearse, 1954) as
ell as by various extractions. Thus the L_1 bodies contain phospholipids.
ow, their reaction to the PE test is not completely negative: the L_1 bodies in
ese preparations appear as blue granules, though they are much reduced in
e. The appearance of the L_1 bodies in PE preparations is very similar to
eir appearance in Hg–BPB, where also they are coloured blue. This clearly

indicates that the L_1 bodies have a core rich in proteins, surrounded b[y a] thick sheath of phospholipids. The core also seems to contain phospholip[ids] in addition to proteins, as L_1 bodies do not appear as rings either in Sudan [or] AH preparations (fig. 1, D; 3, A).

The L_2 bodies, on the contrary, do not have any proteins in them, as th[ey] give a completely negative reaction in Hg–BPB and PE control. They app[ear] much reduced in the AH test. A similar picture is given by the L_2 bodi[es] when the acetone-extracted material is coloured with Sudan black B. T[his] shows that they have a phospholipid core which is surrounded by a sheath [of] some neutral lipids (acetone-soluble) (compare fig. 3, B with fig. 1, H, I).

This duplex nature of the L_2 bodies is further elucidated when AH p[re]parations are coloured with either Sudan III, IV, or Fettrot 7B. In su[ch] preparations the L_2 bodies show a blue-black core (AH positive) surround[ed] by a brilliant pink sheath (Fettrot- or Sudan-positive).

The reactions of the L_2 bodies show that they have a neutral lipid shea[th] which in the absence of other lipids, presumably consists of triglycerides.

Thus it appears that the L_1 bodies contain a core rich in proteins mix[ed] with phospholipids, surrounded by a thick sheath of phospholipids on[ly], whereas the L_2 bodies have a core of phospholipids surrounded by a thi[n] sheath of triglycerides. The lipids of the L_1 bodies are comparatively saturate[d], whereas the lipids of L_2 bodies are highly unsaturated (PFAS positive).

The cytoplasm of the syncytial follicular epithelium also contains lip[id] spheres, which contain phospholipid but no protein.

Vacuoles. The vacuoles present in the cytoplasm do not give a positi[ve] reaction to any of the histochemical tests tried. Thus their contents appe[ar] to be devoid of any lipids, proteins, or carbohydrates. They remain unchang[ed] even in the oldest oocyte studied.

Discussion

The egg of the earthworm is alecithal, since as no yolk globules, consisti[ng] of proteins or carbohydrates or both, have been described in it. The egg of [*P.*] *posthuma* contains three types of well-defined cytoplasmic inclusions, v[iz.] mitochondria, lipid bodies, and vacuoles; the latter are thrown towards t[he] centrifugal pole when the eggs are centrifuged.

Mitochondria. We confirm the observations of Nath and Bhatia (1944) th[at] the mitochondria in the oocytes of *P. posthuma* are granular in form, an[d] remain so throughout oogenesis. Our studies under the phase-contrast an[d] interference microscopes have very clearly shown that the mitochondria, [in] this material, are not filamentous in form. They appear for the first time in th[e] form of a mass near the nucleus. This fragments into several patches and soo[n] the mitochondria get distributed uniformly in the cytoplasm.

Lipid bodies. Nath and Bhatia (1944) described two kinds of lipid granul[es] in the oocytes of *P. posthuma*: the small so-called Golgi granules not stai[n]able with Sudan IV, and spherules colouring with Sudan IV. By employin[g] various Golgi techniques, these authors took great pains to show that th[e]

o-called Golgi granules grow into the spherules colouring with Sudan IV. Ve have also concluded that there are two types of sudanophil lipid spheres 1 the oocytes of the earthworm, viz. L_1 and L_2 bodies, which respectively orrespond to the Golgi granules and Sudan IV spherules of Nath and Bhatia.

Nath and Bhatia (1944) made some observations on the chemical composiion of these lipid bodies, which are not fully warranted by the inadequate Golgi techniques that they used. Nevertheless, with the techniques they mployed, these authors showed: 'To begin with the Golgi granule seems to e made up of a thick cortical part consisting of phospholipides linked with roteins and a very small core consisting of fats or lipoids or both. On account f these proteins in the thick cortical part, the Golgi granule resists solubility n acetic acid and alcohol, and does not stain with Sudan IV. With the growth f the oocyte the Golgi granules also grow, and during their growth they get horn of their protein constituents. Consequently, the cortex of the Golgi esicle is greatly attenuated. The large spherule thus formed differs markedly rom the initial Golgi granule as shown by its stainability with Sudan IV nd its almost complete solubility in acetic acid.'

By employing various modern histochemical techniques we have shown hat the L_1 bodies (Golgi granules of Nath and Bhatia) have, on the contrary, a core of proteins and phospholipids and a cortex of phospholipids only. The L_2 bodies, which are derived from the L_1 bodies by growth and change in hemical composition, have a phospholipid core surrounded by a triglyceride heath. The L_1 bodies resist extraction in pyridine after fixation in weak Bouin (Baker, 1946) owing to their protein constituents; on the contrary, the L_2 bodies are completely dissolved out.

It may be pointed out that in the cockroach egg (Nath and others, 1958) he L_2 bodies contain a core of triglycerides and a sheath of phospholipids. In oth cases the appearance of the L_2 bodies is ringed in osmium techniques Nath and Mohan, 1929, in the cockroach; Nath, 1930, and Nath and Bhatia, 1944, in the earthworm). As pointed out by Nath and others (1958) n the cockroach egg, this ringed appearance of the lipid bodies is due to an ncomplete reduction of osmium tetroxide.

We have shown that the ringed appearance of the L_2 bodies of the egg of he earthworm under the phase-contrast microscope and also under the interference microscope at a particular setting of the analyser is an optical artifact.

It will be clear from this discussion that, since the lipid bodies in the egg of he earthworm have neither a reticular structure nor a fixed chemical composiion, the classical reticular Golgi apparatus is not homologous with these ipid bodies.

Vacuoles. Norminton (1937) described in the egg of *Lumbricus* a heavy luid substance, which goes to the centrifugal pole in the ultracentrifuged eggs. Nath and Bhatia (1944), by employing an ordinary electric centrifuge, conirmed this observation of Norminton. It is significant that Nath and Bhatia 1944) figure and describe this heavy substance as honey-combed in Da Fano, Kolatchev, and even in Bouin preparations. Indeed, in some of the figures

(e.g., text-fig. 6, D) of these authors, delicate, clear vacuoles have been shown in the centrifugal pole occupied by the heavy substance.

Nath and Bhatia (1944) considered these vacuoles as artifacts. We have seen clear delicate vacuoles uniformly distributed in the living oocytes studied under the interference microscope, distinct from the other cytoplasmic inclusions. It has already been stated that under phase-contrast these vacuoles are mixed up with the out-of-focus images of the lipid bodies, which also appear as vacuoles. We have further shown that in all the histochemical preparations, these vacuoles are distinct from the mitochondria and the lipid bodies; they do not contain any lipids or carbohydrates or proteins.

O'Brien and Gatenby (1930) described in the egg of *Lumbricus* vacuoles staining vitally with neutral red, distinct from the mitochondria and the Golgi elements (our lipid bodies). The vacuoles described by us are homologous with the 'vacuolar system' of these authors. It is also significant that Norminton (1937) suggested that the heavy substance is present in the form of droplets in uncentrifuged eggs.

Nucleolar extrusions. Srivastava (1952, 1953) is the only worker on the earthworm egg who has described nucleolar extrusions in *Lumbricus*. The nucleolar extrusions of Srivastava are in fact the protein cores of the L_1 bodies which resist solubility in Bouin and Carnoy.

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Observations on the Developmental Cytology of the Fundic Region of the Rabbit's Stomach, with Particular Reference to the Peptic Cells

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With two plates (figs. 3 and 4)

Summary

1. The first oxyntic cells, differentiating from a non-mucoid cell, appear on the 23rd day of the foetal life of the rabbit.
2. Bowie-positive granules appear in most epithelial cells during foetal life, reach a maximum on the 27th day, and then almost disappear before birth.
3. The first peptic cells containing Bowie-positive granules appear a few hours after birth. They increase after the end of the first week until the adult condition is reached.
4. During the first 4 weeks of postnatal life the pepsinogen granules contain a mucinogen component. This is lost between the fourth and sixth weeks. The loss starts in cells at the base of the glands and proceeds up the tubules towards the crypts.
5. Pepsinogen granules containing a mucinogen component are strongly eosinophil.
6. The first 'mature' peptic granules, that is ones that are Bowie-positive, PAS-negative and non-eosinophil, appear at the fourth week of postnatal life.
7. Mucous neck-cells (of Bensley) begin to appear at the end of the sixth week of postnatal life. Their development is being further investigated.

Introduction

When, in 1932, Plenk wrote his authoritative work on the human stomach, there was no general agreement as to the origin of the principal cell-types in the gastric mucosa. Since that date several papers have appeared dealing with various aspects of the histogenesis of the mucosal cells, but the problem is no nearer being settled. In view of the confusion, the chief purpose of the present investigation has been to elucidate the origin of the main cell-types by using more specific stains and histochemical methods than have been used by earlier writers.

Concerning the embryology of the gastric glands, Kirk (1910), using Bensley's three-colour stain, neutral gentian, and Mayer's muchematein on sections of pig embryos, showed that the oxyntic cells appear at a very early stage (3 cm; birth being at 29 cm), and that they arise from an undifferentiated epithelium 'before any trace of mucus appears'. He states also that although they continue to arise from undifferentiated cells for some time they also 'almost immediately' reproduce themselves by mitotic division. He states that peptic cells develop at 20 cm; they also arise from non-mucous cells and that they show frequent mitoses in the foetus. He writes that he finds no genetic

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relation between oxyntic cells, and peptic or mucous cells. Lim (1922), who examined new-born and foetal cats, stillborn children, and one four-month human foetus, using Mallory's stain, gives the following results. The foetal cats show an epithelium 'entirely devoid of mucus', and at birth glands are present lined by oxyntic and mucoid cells. A week later the glands are larger, oxyntic cells more prominent, mucoid cells present in large numbers, and 'a few developing peptic(?) cells are visible'. These show no mucoid reaction. The human foetus shows mucoid cells and non-mucoid cells, and oxyntic cells are absent. At birth (i.e. the next stage he examines), peptic and oxyntic cells are fully developed. From these results he draws the following unjustifiable conclusion: 'It is quite clear that the gastric glands are in the first instance formed of non-mucoid cells. Later these cells become mucoid in character *throughout the whole stomach*.' The next type to differentiate is the oxyntic, and at a later stage still comes the peptic. Zimmerman (1925), using a $5\frac{1}{2}$-month human foetus and staining his sections with mucicarmine, could find only mucoid and oxyntic cells. As he states that oxyntic cells arise from an undifferentiated epithelium, one must suppose that the peptic cells develop out of the mucoid cell. Plenk (1931) says that peptic cells are present in the child born at full term, and that they definitely develop from an indifferent cell and not from a mucoid cell. Plenk (1932) in his review reaffirms this statement and says emphatically that he agrees with Kirk (1910) that both peptic and oxyntic cells arise from undifferentiated cells. He accepts the results of Lim (1922) quoted above, but does not agree with his conclusions. With regard to Zimmerman (1925) who found only oxyntic and mucous cells in one $5\frac{1}{2}$-month human foetus, Plenk appears not to accept the statement, for, as he says, he can himself see undifferentiated cells in both 5- and 7-month human foetuses.

It is unfortunate that in the most recent paper that I have been able to find on the embryology of the gastric mucosa (Kammeraad, 1942), no positive statement is made as to the precursors of oxyntic and peptic cells. Using embryonic and new-born rats he says that the cells lining the pits and the surface epithelium stain with mucicarmine at birth, but he makes no comment on the cells lining the 'evaginations from the bottoms of the pits' (i.e. the primitive glands), except to state that they are present at this time. He states that oxyntic and peptic cells are both present (presumably in these glands) 24 h later. He writes that only a few zymogen granules are present in these early peptic cells and that these granules are not present in significant numbers until 15 days after birth.

Of the above writers one says that oxyntic cells develop from mucoid cells, whilst three say they arise from non-mucoid cells. Two say the peptic cells develop from mucous cells, and two state they arise from undifferentiated cells, and one writer produced no evidence to support his views (Lim, 1922).

The presence of hydrochloric acid and of rennin (Dudin, 1904) and of pepsin (Keene and Hewer, 1929) may be demonstrated in the fourth and fifth

onths in the stomach of the human foetus and 'consistently thereafter'. erner (1948), studying staining reactions and peptic activity of extracts om the stomach walls, suggests that infants born prematurely may produce nsiderably less pepsin than those born at full term. Hammarsten (1874) und pepsin in the rabbit only after the end of the first week of postnatal life. ecently Hirschowitz (1957), quoting various writers, states that peptic tivity is present in sheep and bovine embryos and in the rat at birth, and at it is not present until one to two weeks after birth in the dog, cat, pig, d rabbit.

Materials and Methods

The period of gestation in those rabbits used in the investigation, that came term, was 30 days $\pm$ 12 h.

Twenty-six foetuses were used. Groups (of never less than 3) were examined two-day intervals from the 19th to the 29th day (inclusive) of gestation. hirty new-born rabbits were used. These were killed in groups of three at e end of 1 day, $3\frac{1}{2}$ days, and 1, 3, 4, $4\frac{1}{2}$, 5, $5\frac{1}{2}$, and 6 weeks after birth (six eing killed at the latter date). The stomachs from four adult rabbits were so examined.

istochemical and cytological techniques

Except with some of the foetuses, where the whole of the mucous membrane as examined, small pieces of the mucous membrane from the greater curvare of the stomach, directly opposite the entrance of the oesophagus, were nned out on cork and subjected to the following procedures:

A. For aldehyde groupings: Th periodic acid/Schiff test, (1) alone,) after incubation in 0·25% diastase, (3) after digestion with trypsin, (4) ter digestion with pepsin, (5) after fat extraction with a boiling mixture of ethanol and chloroform.

B. For glycogen: Bauer's test, (1) alone, (2) after incubation with diastase.

C. For pepsinogen granules: Bowie's method (1936), (1) alone, (2) after t extraction as above.

D. For lipids: (1) Sudan black (for paraffin sections, Thomas, 1948),) alone, (*b*) after digestion with trypsin. (2) For phospholipids, a modificaon of Baker's acid haematein test for use with paraffin sections (Rennels, 951, 1953).

E. By various routine methods and by Altmann's acid fuchsin, a method hich demonstrates early oxyntic cells almost as distinctly as Sudan black.

F. By phase-contrast microscopy on formaldehyde-fixed frozen sections in edia of various refractive indices from 1·33 to 1·656. This method was also epeated after 2 hours' digestion in 0·1% trypsin or pepsin.

Examination by the phase-contrast microscope and methods involving igestion with trypsin or pepsin were carried out only on the 27-day foetus. Methods involving fat extraction were applied only to the 27-day foetus and

to the animals killed 3 weeks after birth; with these exceptions all the abo techniques were used on all specimens.

Although Bowie's method (1936) referred to above stains nothing b pepsinogen granules in the adult gastric mucosa, it also stains the pancrea zymogen granules, and therefore no histochemical specificity can be claim for it.

Results

The adult fundic mucosa

The surface epithelium of the stomach dips down into crypts (fig. 1). T gastric tubes are arranged perpendicularly to the surface of the mucosa a penetrate its whole thickness. They are simp branched tubules which open either singly in groups of two or three, through a constri tion into the bottom of the crypts. The blin ends are slightly thickened and coiled, a sometimes divide into two or more branche In textbooks the tubules are frequently d scribed in three sections. These are the nec the body, and the base (fig. 1), which almo reaches the muscularis mucosae.

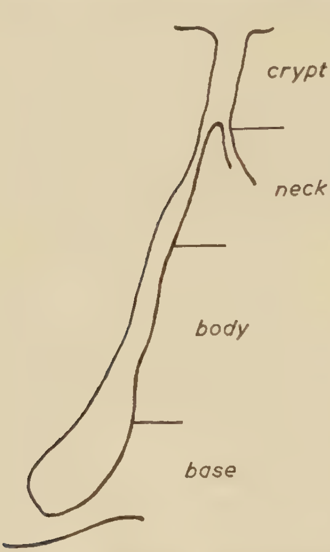

Fig. 1. A diagram of a gastric gland from the stomach lumen (above), down to the base near the muscularis mucosae.

The surface epithelium. This is a simp columnar epithelium. It is very regular an contains no goblet cells such as were describe in the pig by Kirk. The cells on the surface the stomach are frequently somewhat talle than those in the bottom of the crypts. Th nucleus is ovoid and placed towards the base ment membrane. Numerous granules, givin a PAS-positive reaction, fill the supranuclea part of the cells. In many cases in cells furthe down the crypts the granules are more nearl confined to the luminal region of the cytoplasn at the free border of the cells, but in other cases there is no difference betwee surface and crypt epithelial cells. There is no Bowie-positive material i any of these cells.

The oxyntic cells. These cells are easily recognizable, even in unstaine sections, by their large size and their spherical or triangular shape. They hav a readily visible cell border and possess an ovoid, centrally placed nucleus They have a granular cytoplasm and intracellular canals are occasionall visible even when special techniques are not employed. Oxyntic cells ar plentiful in the bodies and bases of the glands, but are more numerous in th neck region. I have seen them in the crypts and between cells on the surfac epithelium, but in the adult this is a rare curiosity.

The peptic cells. With Bowie's method these are at once recognizable by th

p blue colouring of the pepsinogen granules that they contain. The cells upy the bodies and the bases of the glands, becoming more numerous as y approach the base.

he mucous neck-cells (*of Bensley*). These cells very rarely lie adjacent to ther but are placed between the oxyntic cells in the neck region of the nds, and frequently extend down as far as the uppermost peptic cells. I e never seen them in the bodies or bases of the glands in the rabbit. Except ere two lie next to each other, when they are roughly cuboidal in shape, y are the most irregularly shaped cells in the mucosa. This is probably due heir being slotted between adjacent oxyntic cells. Although their shape is gular, it is certain that part of the cells touches the basement membrane does part of the oxyntic cells). They have flattened nuclei occupying the es of the cells, and their cytoplasm is filled with numerous fine PAS-itive granules.

e fundic mucosa in the foetus

The 19-day foetus (*3 specimens*). A tall columnar epithelium lines the fundic a of the stomach. The surface undulates slightly, and here and there forms -like evaginations towards the underlying mesenchyme. None of the adult -types already mentioned can be seen. There is no PAS-positive material sent. (This is in contrast to the large amount already present in the form granules and ring-like structures in the cells of the pyloric region.)

The 21-day foetus (*3 specimens*). More pits are present in the mucosa, ough there is, as yet, no sign of tubules. The PAS test shows a few granules l rings in the supranuclear region of a few cells. A few primitive blood-cells our with Bowie's method, otherwise the method is negative.

The 23- and 25-day foetuses (*8 specimens*). PAS-positive material in the m of granules and sometimes rings is present in the supranuclear region of ny cells, both surface cells and those lining the pits. Bowie's method shows nin line of granules close to the free border of the cells, in similar situations.

Occasional oxyntic cells are seen in some of the embryos, usually situated he bottom of the pits, but never situated near cells containing PAS-positive terial. So far as the above results are concerned, it is impossible to dis-guish between the 23rd and 25th day.

The 27-day foetus (*6 specimens*). More pits are present in the epithelium n at earlier stages. Very occasionally a constriction appears in a pit towards base, the lumen being narrower below this point. This is probably the first n of the future crypts and tubules of the adult.

Oxyntic cells are much more numerous, two or three sometimes lying along-e each other. They are somewhat more frequent at the bases of the pits than y are along their sides.

All the cells of the epithelium, other than oxyntic cells, contain granules ich are PAS-positive (figs. 2, A; 3, A). These granules are more abundant the cells lining the stomach lumen than they are in the cells lining the pits. ey are present in large quantities at the free borders of the cells. An area

clear of granules is left immediately above the nucleus. Other granules, l numerous, occupy a position alongside the nucleus. It is doubtful if any PA positive material occurs below the nucleus. Where it seems to do so it appe to be in an underlying cell or in a cell cut obliquely. After digestion w trypsin or pepsin and after fat extraction, both supra- and paranuclear grou of granules are still PAS-positive.

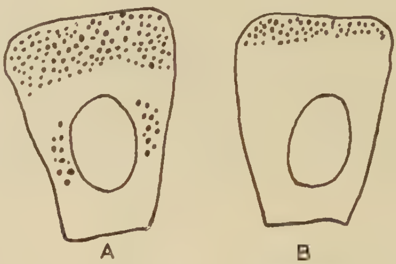

FIG. 2. A diagram of two epithelial cells.
A, PAS. B, Bowie's method.

All cells, except oxyntic cells, after Bowie's method contain some brig blue granules (figs. 2, B; 3, B). As with the PAS technique, the granules a more numerous in the cells lining the stomach lumen than in those lining t pits. The granules again occupy a supranuclear position at the free borders the cells, being less numerous than the corresponding PAS-positive granul No blue granules, however, are ever seen in a paranuclear position (compa figs. 2, A, B), either before or after fat extraction.

A few supranuclear and many paranuclear sudanophil granules are prese in the cells that have just been described. The granules are spherical exce alongside the nucleus, where many appear elongated.

Both the supranuclear and paranuclear granules (described as PA positive) can be seen by phase-contrast microscopy. There is no difference clarity between the two groups of granules, both being clearest in a mediu of refractive index 1·43. In media with refractive indices above 1·481 no can be seen. Both groups are also visible after digestion with trypsin and peps and after fat extraction.

The 29-day foetus (*6 specimens*). PAS-positive granules are visible in a cells, except the oxyntic, as at the 27-day stage, but most of the cells show a increase in quantity in the supranuclear position. In many cells at the base the tubules the PAS-positive material appears foamy, a condition seen on in the adult in the mucous neck-cells after formaldehyde fixation.

Bowie-positive granules are far less numerous than in the 27-day embry the great majority of the cells showing none at all. Here and there, a group c a few cells either on the stomach lumen or deep in the pits and tubules sho some in a supranuclear position. Some of these cells show only a single lin of granules at their free borders.

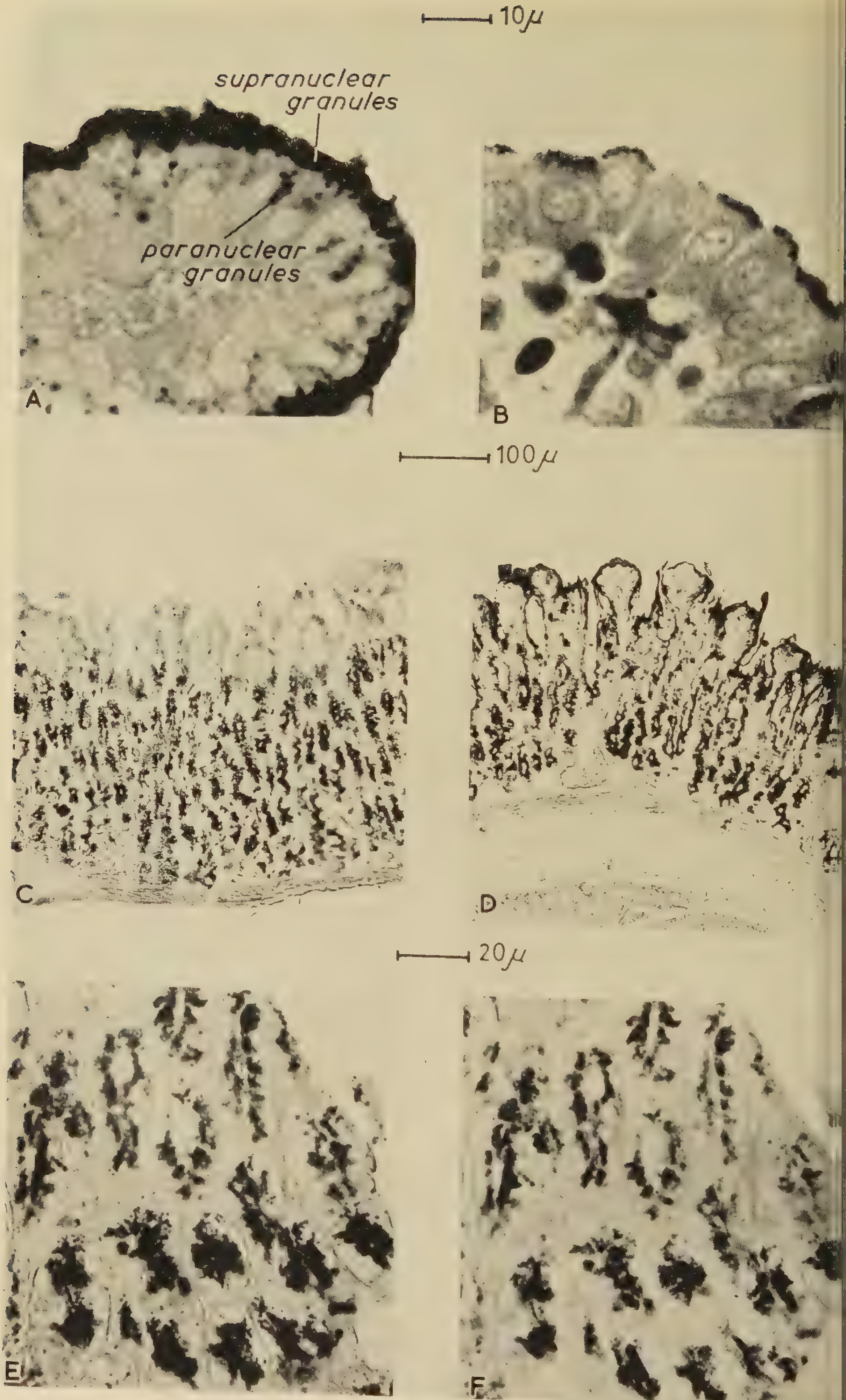

FIG. 3

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he fundic mucosa in the new-born rabbit

One day old (*3 animals*). All the cells except the oxyntic show much PAS-positive material in the form of granules and ring-like structures. In addition the supranuclear and paranuclear groups that were present as in the embryo, some granules and many rings are situated in the basal part of the cells below the nucleus. The rings are frequently quite large, presenting a similar picture in this respect to the cells in the pyloric glands in the 19-day foetus.

None of the cells along the stomach lumen or in the upper third of the pits show any granules that colour with Bowie's method. But, in the lower two-thirds of some of the pits and tubules, and especially in the basal portions, some cells contain greyish-blue granules after Bowie's method. These cells are not numerous but occur here and there in all sections from all animals. Cells that contain these blue granules can also be shown to contain PAS-positive granules.

Three-and-a-half and seven days old (*6 animals*). There are more oxyntic cells than previously and an occasional intracellular canal can be seen in some of them. The oxyntic cells are as numerous along the bodies of the tubules as at their bases. Only one was seen on the surface of the stomach.

PAS-positive granules are seen in all cells except oxyntic.

The Bowie method shows greyish-blue granules in a few cells. Again these cells are situated in the deeper half of the tubules and are few in number, it being doubtful if there are many more present than in the 1-day-old rabbits. Not only do these cells contain Bowie-positive granules and PAS-positive granules, but it can be shown to be the same granules that colour with both techniques. This fact was established by colouring with Bowie's method, choosing cells, decolorizing, and examining them again after they had been subjected to the PAS technique.

Three weeks old (*3 animals*). Many more oxyntic cells are present. Bowie's method (fig. 3, C) shows many cells containing greyish-blue granules. These cells are in many sections mainly in the bases and bodies of the tubules, but other sections show them frequently extending into the neck of the tubules (fig. 3, C), right up to the base of the crypt.

The PAS technique (fig. 3, D) shows positive granules in probably all cells

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FIG. 3 (plate). A, 27-day foetus. PAS. Shows supranuclear and paranuclear groups of granules.

B, same as A. Bowie's stain. Shows supranuclear granules. (Note that immature red blood-cells also colour.)

C, 3-weeks-old animal. Bowie's stain. Shows granules in peptic cells lining the gastric tubules from the bases of the glands up to the crypts.

D, 3-weeks-old animal. PAS. The granules in the peptic cells, the surface epithelium, and the epithelium of the crypts are coloured.

E, 3-weeks-old animal. Bowie's stain. For comparison with F.

F, 3-weeks-old animal. PAS. This is the *same* section as E, with Bowie stain removed by alcohol before the PAS method was applied. The photomicrograph shows that the granules that colour with Bowie's method are the same as those colouring after PAS.

of the mucous membrane, except the oxyntic cells. Again the granules t colour blue by Bowie's method are PAS-positive (figs. 3, E, F). Further, it v observed that these same granules are very strongly eosinophil. They are sudanophil, nor do they show the presence of phospholipid.

These granules do not colour by Bauer's method and they remain PA positive both after incubation in diastase and after immersion in boil methanol and chloroform.

Four weeks old (*3 animals*). Apart from a marked increase in the number cells present in the 4-weeks-old animals, similar results are obtained, exc that there are small groups of cells in the basal regions of adjacent tubules t contain no PAS-positive material (fig. 4, A, arrow). Some of these are oxyn cells, and the rest can easily be shown to contain Bowie-positive granul This is the first appearance of a cell which contains Bowie-positive granu that are not at the same time PAS-positive. Further, the granules which PAS-positive are a greyish blue after Bowie's method, whereas those whi are PAS-negative are a bright blue after Bowie's method. Moreover, t former granules which are both PAS-positive and Bowie-positive, colo intensely with eosin, whereas the latter granules which are PAS-negative b Bowie-positive are not eosinophil.

From 4½ to 5½ weeks old (*9 animals*). The groups of PAS-negative cells the basal region described above become progressively larger and mo frequent until in some of the 5½-weeks-old animals there are almost no PA positive granules in the lower one-half to two-thirds of the tubules (fig. 4, At this stage the granules which remain PAS-positive in the upper half one-third of the tubules colour a greyish blue with Bowie's method, where granules in peptic cells in the lower one-half to two-thirds of the tubu colour a bright blue after this method.

There is some variation in the nine animals described, but generally spea ing an animal in one *age group* shows similar results to one of equal *size* a *weight* in another age group. Also, in some individual sections where pep granules are PAS-negative in the lower half of the tubules, here and there small groups there are tubules whose 'peptic' cells from crypt to base sho PAS-positive granules.

Six weeks old (*6 animals*). The cell-types and their distribution are almo

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FIG. 4 (plate). A, 4-weeks-old animal. PAS. Shows many PAS-positive peptic granules cells lining the tubules, and an area (arrow) in the base where there are no PAS-posit granules. This area, however, was filled with Bowie-positive granules (see text).

B, 5½-weeks-old animal. PAS. Shows that at this stage (compare A) the mucinogen co ponent of the peptic granule (PAS-positive) is lost in many cells in the lower half of the gast glands.

C, an animal from the 6-weeks-old group. PAS. Shows that the vast majority of cells co taining PAS-positive granules are now in the surface epithelium, in the epithelium of t crypts, and in a layer of cells in the neck region.

D, the same animal as C. Bowie's stain. Shows not only that Bowie-positive granules present in peptic cells along the whole length of the tubules, but also that the cells that PAS-positive in the neck region of C contain Bowie-positive granules as well.

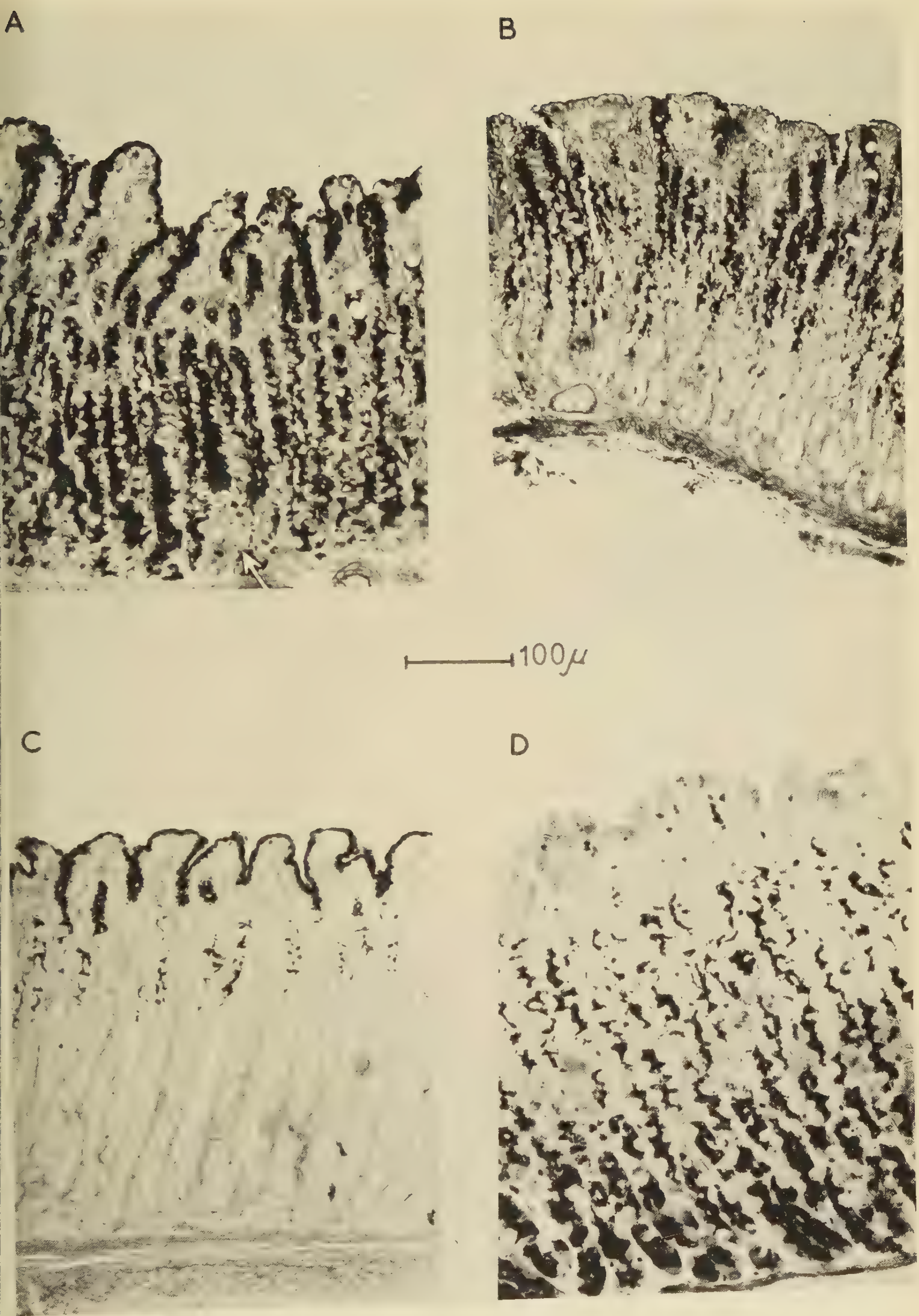

FIG. 4

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ilar to the adult's, though the mucosa is only one-third of its full depth. four animals, Bowie-positive granules are found in cells higher in the neck g. 4, D) than in the adult and these granules are PAS-positive (fig. 4, C) and ongly eosinophil. Typical mucous neck-cells are still few in number in all ecimens.

Discussion

In discussing the origin and development of cell-types in the gastric mucosa appears that some authors have adopted the presumption that a cell arises om the same type of cell during embryonic development as during normal placement or regeneration in the adult. For example, Hirschowitz (1957), ider the heading of 'embryology', states that peptic cells arise from mucous ck-cells, and quotes Plenk (1932) as an authority. Plenk (1932, p. 143) rtainly wrote that in the fully adult stomach the replacement of peptic cells ust occur through the differentiation of mucous neck-cells, and one must sume that this is the basis for Hirschowitz's statement. Plenk also wrote, wever (ibid., p. 101), that peptic cells develop in the embryo from an unfferentiated non-mucoid cell, reaffirming his own investigations of 1931 and ose of Kirk (1910). It appears therefore that this presumption is not necesrily true, and it becomes essential to study the development in the embryo self without preconceived ideas derived from regenerative phenomena in the lult.

In the foetal stages in the rabbit the results show that the oxyntic cells evelop from undifferentiated non-mucoid cells about the 23rd day. This findg agrees with the observations of Kirk (1910) and of Plenk (1932), but is not agreement with the statements of Lim (1922).

At the 27th day there are PAS-positive granules in all cells except the xyntic. These are usually arranged in two groups, supranuclear and parauclear. Sudanophil material is conspicuous in the paranuclear group, where appears to be applied around some of the granules. Since all the PASositive granules are still positive after incubation in diastase and after chlororm extraction, when the sudanophil material is absent, they are presumably mucinogen and neither glycogen nor glycolipid. Phase-contrast microscopy ows no difference between the groups of granules, nor can any difference be emonstrated by peptic or tryptic digestion. They do not digest, but this may e due to the special method of fixation and prolonged postchroming.

Bowie-positive granules appear in cells at the 23rd day; they increase to eir maximum per cell at the 27th day, and then decrease rapidly and are uch less numerous in the last days of foetal life, when many sections show one at all in the whole length of the epithelium. They always occupy a upranuclear position, and even at the 27th-day stage there are never as many er cell as there are PAS-positive granules. Phase-microscopy of PASections does not reveal any granules in a supranuclear position other than AS-positive ones; therefore it is almost certain that the Bowie-positive ranules are in fact PAS-positive.

In view of what has just been said, the question arises whether the Bowie-positive granules are in fact pepsinogen, and if a temporary stage of peptic activity occurs during foetal life which later diminishes, or disappears, to reappear in postnatal life. Unfortunately this question cannot be answered at the present moment, for two reasons. In the first place, no evidence has been provided to indicate that pepsin is present in the stomach of the rabbit before the end of the first week of postnatal life. But at the same time it must be borne in mind that various writers in the past have given widely differing dates for the onset of peptic activity in other mammals (Keene and Hewer, 1929). In the second place, as already indicated, it cannot be asserted that Bowie's method is specific for pepsinogen. Further, it is not even possible to say if the future peptic cells develop from the cells that contain Bowie-positive granules in the foetus, since in any case just before birth the majority of the undifferentiated epithelial cells show no Bowie-positive granules. It might, however, be relevant to mention at this point that the foetal Bowie-positive granules are PAS-positive also, as are in fact the peptic granules in the first few weeks of postnatal life. This might be considered as circumstantial evidence in support of the notion that the Bowie-positive cells of the foetus are indeed a special sort of peptic cell.

At birth, routine staining methods demonstrate a few cells in the bases of the tubules which all previous writers have called peptic cells. I shall continue to call them peptic cells, for they are present when pepsin can be found in the stomach, but, as they also contain PAS-positive granules, it must be borne in mind that some, if not all, may prove to be 'stem cells', that is to say cells from which not only adult peptic cells but other cells may develop.

From birth to the fourth week no granules can be seen in any of the peptic cells other than PAS-positive ones (and these are also Bowie-positive—see below). Between the fourth and sixth weeks the granules lose their PAS-positive reaction. This loss commences in cells at the base of the tubule, and proceeds upwards towards the lumen until approximately the end of the 6th week, when the adult condition is reached; there is then no PAS-positive material in the peptic cells. Although, unfortunately, exact physiological data are lacking, this loss of the mucinogen component of the peptic granules does not occur at the end of the first or second week, when it is said that peptic activity can first be detected in the rabbit (Hirschowitz, 1957). It is of interest to note that the animals used were weaned during this period of 4 to 6 weeks after birth.

The number of peptic cells present at birth and containing Bowie-positive granules remains approximately constant up to the end of the first week and then steadily increases until the adult condition is reached. The peptic granules, which are PAS-positive, colour a greyish blue after Bowie's method, whereas those that have lost their mucinogen component colour a bright blue.

The results establish without doubt that the peptic cells, in the rabbit, not only differentiate from cells containing mucinogen (as was in my opinion demonstrated by Zimmerman, 1925) but retain a mucinogen component for

veral weeks. This does not agree with Kirk's statement that the peptic cells ise directly from primitive embryonic cells and not from mucous cells. This screpancy between Kirk's observations and those reported here may have een due to the fixative he used, a mixture of mercuric chloride and potasum dichromate in alcohol for only 2 h, or perhaps there is a species difrence, for he used pig embryos. Again Plenk, writing of human embryos, ates that the peptic cell definitely develops from an indifferent cell and not om a mucoid one. This I imagine was due to the paucity of methods availble, for I have noticed in a 3-weeks-old child that peptic cells contain Bowiesitive granules which can easily be shown to be PAS-positive also, and rther, in a 5-months-old Rhesus monkey foetus numerous Bowie-positive ranules are present in the peptic cells and these are PAS-positive also. It may e of interest to note here that in some species I have examined, for example, t, dog, and fennec fox (*Fennecus zerda*), the adult peptic cells contain a few AS-positive granules, whilst in adult guinea pigs it would appear that most not all the pepsinogen granules are both PAS- and Bowie-positive.

Fergusson (1928) continuing the work of Griffini and Vassale (1888), Cade 901), Harvey (1907), and Malesani (1909) found that both peptic and oxyntic ells can arise from mucoid cells. However, neither Fergusson nor any of these vestigators was studying the development of the foetus: all were investigating e repair of traumatic injuries to the adult mucous membrane. Although it ems there is no difference between normal development and regeneration llowing trauma in regard to peptic cells, this is not true of oxyntic cells.

I would like to thank Mr. Charles Voyle, senior technologist of the Department of Anatomy, St. Mary's Hospital Medical School, for taking the photomicrographs for me.

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The Chromosome Numbers of certain Barnacles in British Waters

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With two plates (figs. 2 and 3)

Summary

The chromosome numbers of nine species of sessile barnacles have been determined from squashes of young embryos stained by an iron alum aceto-carmine method. All the species of *Balanus* examined, and *Elminius modestus* had a diploid count of 32 chromosomes. *Chthamalus stellatus* and *Verruca stroemia* had each 30 chromosomes. Meiosis occurs after the egg passes into the mantle cavity, and the cytological changes accompanying the extrusion of the two polar bodies are figured.

Introduction

During the last 40 years a number of papers have been published dealing with the nuclear cytology and chromosome number of several species of Crustacea, including members of most of the major groups. Very little cytological work, however, has been published on the Cirripedia. This paper gives the chromosome counts and some details of the nucleus of nine species of the commoner barnacles found in British waters.

Chromosome numbers vary considerably among the different groups of Crustacea. In Branchiopoda it is particularly variable, ranging from $2n = 8$ in three species of *Tripos* (Longhurst, 1955) to $2n = 84$ in *Artemia salina* L. (Artom, 1929). The Copepoda generally give rather small diploid counts of 12 to 14 (Stella, 1931; Heberer, 1927). In Amphipoda the counts vary from 26 to 54 (Orian and Callan, 1957); in Isopoda from 16 to 24 (Vandel, 1926). In the Decapoda the chromosome number is very high, four species of Brachyura having 94 to 124 (Niiyama, 1942) and the anomuran *Eupagurus ochotensis* 254 chromosomes (Niiyama, 1951).

In the Cirripedia, if we ignore the approximate haploid count of 4 to 12 made by Groom (1894), chromosome counts have been made only in two species, both belonging to Lepadidae. They are *Lepas anatifera* (L.) which has been reported as having 26 chromosomes (Witschi, 1935), and *Scalpellum scalpellum* L. with 32 chromosomes (Callan, 1941).

Methods

Mitotic and meiotic divisions were studied in alum acetocarmine squash preparations made from developing embryos and oocytes in the egg masses of gravid barnacles.

The success of the preparation was found to depend upon the stage of

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development of the embryo immediately before fixation. The most satisfactory results were obtained from embryos in which the yolk was almost completely covered by blastoderm, but in which limb-bud rudiments had not formed; that is, stages 4-7 in Crisp's (1954) nomenclature. These stages are usually reached a few days after fertilization. In preparations from the later embryonic stages, particularly those which had developed appendages (i.e. stages 9–13), the nuclei were small and rarely in division. It was important to have available embryos of each of the species to be studied in the correct stage of development. We therefore took advantage of concurrent work on temperature conditioning of breeding in Cirripedes (Crisp, 1957) to provide embryos

TABLE 1

Sources of material

Species	*Season when young embryos may be collected*	*Collecting ground*
V. stroemia (O. F. Müller) . .	Nov.–Dec.	Brixham, S. Devon
C. stellatus (Poli)*	June–Sept.	W. Anglesey, Aberffraw
B. balanoides (Linnaeus) . . .	Nov.	Bangor Pier
B. amphitrite var. *denticulata* (Broch)*	May–Sept.	Swansea, Queen's Dock
B. crenatus Bruguière . . .	Jan.–Mar.	Bangor, Penrhyn
B. hameri (Ascanius) . . .	Jan.	Irish Sea
B. perforatus Bruguière . . .	Aug.–Sept.	Gower Peninsula, S. Wales
B. balanus (L.)	Jan.–Feb.	Beaumaris Bay
Elminius modestus Darwin . .	Mar.–Oct.	Bangor Pier

* Egg masses obtained out of season by maintaining and feeding in warmed sea-water.

from species which were not in the natural breeding condition at the time this work was carried out, viz. November 1957 to January 1958. At this time of year only the embryos of *Balanus balanoides* (L.), *Verruca stroemia* (O. F. Müller), *B. hameri* (Ascanius), and *Elminius modestus* Darwin could be collected in the appropriate stage of development. Egg masses of *B. balanus* (L.) (= *B. porcatus* da Costa) and *B. crenatus* Bruguière had already been fixed during their breeding season early in 1957. The embryos of the southern forms *Chthamalus stellatus* (Poli), *B. amphitrite* var. *denticulata* (Broch), and *B. perforatus* Bruguière were obtained by maintaining the animals at a constant temperature of about 25° C and feeding liberally with planktonic food or *Artemia* larvae. Table 1 gives the time of year when suitable embryos may be found and the collecting grounds of the species studied.

The technique used to demonstrate the chromosomes is essentially that described by Godward (1948). Modifications of this technique, which had been employed successfully in staining the tiny chromosomes of certain members of the Rhodophyceae (Austin, 1956), were equally successful with this animal material. The schedule differed from the basic aceto-carmine technique (Belling, 1926) in that a separate mordant bath of ferric alum was

employed. With barnacle egg masses this procedure has proved superior both to aceto-carmine used alone and also to aceto-orcein.

The aceto-carmine solution was prepared according to Belling (1926) but with the addition of 1 drop of saturated ferric acetate solution per 10 ml of stain (Thomas, 1940). The schedule is summarized below.

1. Fix in 1:3 acetic alcohol, using absolute methyl alcohol. If necessary store fixed material in deep freeze or transfer to 98% alcohol.
2. Wash out fixative by means of several changes of tap water.
3. Steep for 20–30 min in a 1% aqueous solution of ferric ammonium sulphate.
4. Wash in tap water for 20–30 min with frequent changes.
5. Place small pieces of egg mass or single embryos in a drop of aceto-carmine on a microscope slide and heat repeatedly almost to boiling-point. Replenish the carmine and continue heating until the material has turned a purple-black colour; then place a coverslip over the specimen and blot excess stain away. Squash the stained tissue by exerting careful and controlled pressure on the cover glass with a flat-honed ebony instrument.
6. Ring and seal with glycerine jelly. The preparations remain in perfect condition for months and can be made permanent at any time by any of the usual methods (McClintock, 1929; Bradley, 1948).

The above treatment proved uniformly successful when applied to the egg masses of all the nine species of cirripedes investigated. The degree of spreading required to demonstrate the polar body extrusion and meiotic figures in the large yolky eggs of *B. balanoides* needed, however, exceptionally careful control. The chromosome number has been established after examining at least 20 nuclei of each species.

Observations on mitotic divisions

The observations made on the somatic mitosis of the different species of barnacles are summarized in table 2. In most of the species late prophase, prometaphase, and telophase figures were frequent, whilst metaphase and anaphase were less common. The chromosomes could be counted fairly accurately at prophase but were more distinct in prometaphase, becoming somewhat clumped and less suitable again at late metaphase.

From table 2 it can be seen that the chromosome number of all the six species of *Balanus* investigated and of *E. modestus* is $2n = 32$, whilst that of *C. stellatus* and *V. stroemia* is $2n = 30$. Taking into account the earlier counts of 26 chromosomes in *L. anatifera* (Witschi, 1935) and 32 in *S. scalpellum* (Callan, 1941), it seems that the group is as a whole homogeneous and that polyploidy has played no part in its evolution. It is interesting that in its chromosome number *Elminius* resembles *Balanus* and differs from *Chthamalus*, thus supporting Darwin's views as to its inclusion in the Balaninae rather than in the Chthmalinae. Chromosome numbers, however, have only limited value in the taxonomy of cirripedes, though it is possible that a comparative study of chromosome morphology might reveal interesting results.

TABLE 2

Cytological details of nuclei of young cirripede embryos

Species	No. of nuclei examined	Haploid-chromosome number (n)	Diploid-chromosome number ($2n$)	Diameter (in μ) of resting nucleus	Prophase: Average size (in μ) of nucleus (diam.)	Prophase: nucleolus (diam.)	Prophase: chromosomes largest	Prophase: chromosomes smallest	Prometaphase: Average size (in μ) of nucleus (diam.)	Prometaphase: nucleolus (diam.)	Prometaphase: chromosomes largest	Prometaphase: chromosomes smallest
V. stroemia	46	—	30	—	47·1	3·9	5·5	2·8	36·2	—	2·7	1·4
C. stellatus	30	—	30	5·3	28·2	2·5	4·7	2·2	21·6	—	2·4	1·2
B. balanoides	25	16	32	12·7	32·9	2·1	4·9	2·8	29·9	3·4	3·0	1·7
B. amphitrite	25	—	32	7·6	17·0	4·0	3·6	1·8	17·6	2·3	2·4	1·1
B. crenatus	27	—	32	11·7	32·3	4·2	5·6	2·7	26·7	3·8	3·7	2·0
B. hameri	20	—	32	13·0	44·4	4·0	4·3	1·4	38·0	3·7	4·0	1·8
B. perforatus	28	—	32	8·4	26·0	4·3	5·6	3·0	14·8	—	2·1	1·2
B. balanus	30	—	32	10·1	26·1	5·3	4·4	2·4	29·6	3·5	2·4	1·7
E. modestus	34	—	32	9·2	45·0	2·5	8·9	3·1	31·6	—	2·7	1·4

In general the size of the nuclei in the barnacle embryo is small, smaller indeed than those of the amphipods recently investigated by Orian and Callan (1957) with modern methods. The alum aceto-carmine technique, however, results in a greater degree of swelling and spreading of the chromosomes than is caused by aceto-orcein (Griggs, 1946).

The smallest nuclei and chromosomes belong to *B. perforatus* (fig. 2, E) and *B. amphitrite*, the latter having particularly small chromosomes (fig. 1, B). *E. modestus* (fig. 2, G), *V. stroemia* (fig. 2, F), *B. balanoides* (fig. 3, H), and particularly *B. hameri* (figs. 2, B; 1, A) have comparatively large nuclei.

FIG. 1. Nuclei in late prometaphase of mitosis in (A) *B. hameri*, and (B) *B. amphitrite*; drawn to the same scale. Both show the nucleolar-organizing or nucleolar-zone chromosomes.

The appearance of the nucleoli and the changes which they undergo are constant and characteristic in each species. A single nucleolus is present in the early prophase stages of all the species studied. *B. balanus* has an unusually large and persistent nucleolus (fig. 2, D). In *V. stroemia* and *E. modestus* the nucleolus is rarely demonstrable. *B. hameri* has a small persistent nucleolus and, like *B. crenatus*, shows the relationship between nucleolar organizing chromosomes and the nucleolus. It appears that there are two or three chromosomes involved in the formation of the nucleolus (figs. 2, B; 1, A). The latter may break up during prophase into fragments of different size. These remain attached to the nucleolar-organizing or nucleolar-zone chromosomes (figs. 1, A, B; 2, B, C). These chromosomes can also be seen in *B. perforatus*, *B. amphitrite*, *E. modestus*, and *C. stellatus*. They persist until the end of prometaphase in *B. amphitrite*, *B. balanoides*, *B. crenatus*, *B. perforatus*, and *B. hameri*, but disappear at the end of prophase in the remaining four species.

It was possible to discern the position of the centromeres in the late prophase and early prometaphase chromosomes of most of the species. They can be seen in *E. modestus* (fig. 2, A), *B. hameri* (fig. 2, B), *B. crenatus* (fig. 2, C), *B. balanus* (fig. 2, D), *V. stroemia* (fig. 2, F), and *B. balanoides* (fig. 3, H). Median and submedian positions of the centromere are prevalent.

The contraction of the chromosomes from prophase to metaphase has also been measured in each species. The greatest degree of contraction was observed in the chromosomes of *E. modestus* where they became reduced by 2·1 to 3·3 times their original early prophase length by the end of prometaphase. This can be compared with 2 to 2·6 times in *B. perforatus* and *V. stroemia* and only 1·3 to 1·5 times in *B. crenatus*. The remaining species are closely similar to the last with a contraction of from 1·5 to 1·9.

Observations on meiotic divisions

These observations were carried out chiefly on the oocyte nuclei of *B. balanoides* and to a lesser extent on those of *B. crenatus*. For studying the meiotic divisions of the nuclei of *B. balanoides*, specimens collected in the first week of November were watched in the laboratory until copulation and insemination occurred. The penis was seen to be inserted in a neighbouring individual and a white stream of semen observed flowing within the semi-transparent tissue of the penis. The egg masses of the inseminated individual were fixed in acetic-alcohol at hourly intervals from one to 36 h after insemination had taken place.

The formation and loss of the first polar body followed very soon upon oviposition. During the first hour it was possible to see the first meiotic anaphase within the eggs, which were at this time spherical and surrounded by active sperm and sperm tails. The first meiotic anaphase has a well-marked spindle region and a dark cell-plate zone (fig. 3, A). Fig. 3, B shows the extrusion of the first polar body from the still naked egg; this process is figured by Groom (1894) for *B. perforatus*. The first polar body is small, about 10–11 μ in diameter, smaller in fact than the remaining female haploid nucleus, in which the early anaphase of the second meiotic division can be seen. The second division closely follows the first without an interphase (fig. 3, C). The egg then assumes an ovoid shape and the egg membrane separates from the egg itself and forms a conspicuous egg case within which the embryo develops. It seems likely that the sperm nucleus enters the egg before this membrane is fully formed, though this has not been observed.

FIG. 2 (plate). Mitotic division in cirripede nuclei.

A, *E. modestus*: chromosomes in extended prophase state; indistinct nucleolus at top left.

B, *B. hameri*: nucleus at early prometaphase with nucleolus and nucleolar chromosomes clearly shown; $2n = 32$.

C, *B. crenatus*: early prometaphase nucleus showing $2n = 32$ chromosomes and the large but faint nucleolus.

D, *B. balanus*: early prometaphase showing the 32 chromosomes and the large very distinct nucleolus.

E, *B. perforatus*: later prometaphase; $2n = 32$.

F, *V. stroemia*: a well squashed late prometaphase showing the 30 chromosomes.

G, *E. modestus*: late prometaphase to show the 32 chromosomes.

H, *C. stellatus*: very late prometaphase showing the 30 highly contracted chromosomes and a faintly stained interphase nucleus to left.

I, *V. stroemia*: metaphase plate in oblique lateral view; the 30 chromosomes can be easily counted.

J, *V. stroemia*: late anaphase; groups of 30 chromosomes at each pole.

FIG. 2

A. P. AUSTIN, D. J. CRISP, *and* A. M. PATIL

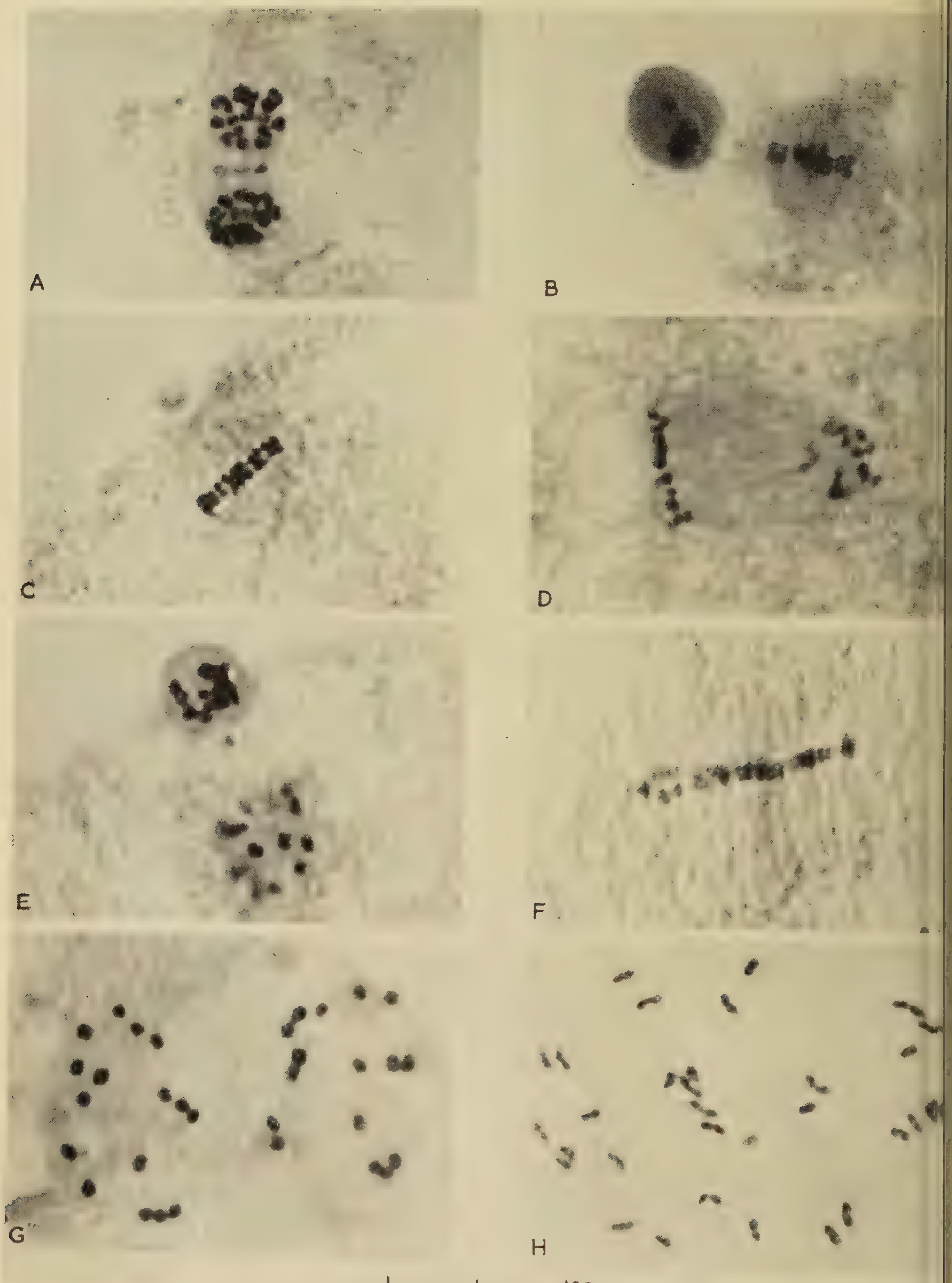

Fig. 3

A. P. AUSTIN, D. J. CRISP, *and* A. M. PATIL

The second polar body (fig. 3, D, E) is formed and extruded inside the fully formed egg case, as described by Groom, where it persists for up to 20 h after insemination. The second polar body has not been seen to divide into two; indeed, the clumping of its chromosomes at the time it is extruded suggests that a further division may not occur. At 14–24 h after insemination the poles of the egg become progressively more conspicuous and at this time the nucleus enters the first cleavage division (fig. 3, F). The first cleavage of the egg usually begins about 20–30 h after insemination, and is closely followed by further divisions. Chromosome counts were made on the female nucleus of *B. balanoides* at late anaphase of the second meiotic division (fig. 3, G). In more than a dozen oocytes the haploid number was established as $n = 16$. This confirms the diploid count of $2n = 32$ in the somatic chromosomes of young embryos of the same species.

We are grateful to Prof. E. W. Knight-Jones for supplying specimens of *B. perforatus* and *B. amphitrite* from Swansea; to Miss E. Clay who supplied *V. stroemia* from Brixham, and to Mr. J. S. Colman and Dr. D. I. Williamson for collecting and forwarding specimens of *B. hameri* dredged from grounds off the Isle of Man.

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FIG. 3 (plate). A–F, sequence of changes during meiosis in *B. balanoides.*

A, first meiotic telophase. The large chromosomes are closely grouped, the spindle region is distinct and the cell-plate zone rather dark.

B, extrusion of the 1st polar body, in which the chromosomes appear to have become clumped. The remaining female haploid nucleus is entering anaphase of the 2nd meiotic division.

C, 2nd meiotic anaphase after loss of 1st polar body.

D, formation of the 2nd polar body. The spindle region and the vesicle which will separate as the polar body can be clearly seen.

E, extrusion of the 2nd polar body. The chromosomes become clumped together as soon as the polar body separates from the egg.

F, 1st cleavage division. Lateral view of the metaphase plate of the 1st division of the diploid (zygotic) nucleus.

G–H, chromosomes of *B. balanoides.*

G, a well squashed 2nd meiotic anaphase, to show the two groups of 16 chromosomes.

H, a well squashed mitotic prometaphase in *B. balanoides* to show the $2n = 32$ number of chromosomes.

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The 'Posterior Lobes' of *Nephtys*: Observations on three New England Species

By R. B. CLARK

(*From the Department of Zoology, University of Bristol*)

With one plate (fig. 2)

Summary

The disposition of the prostomial mucus-glands in *Nephtys picta*, *N. incisa*, and *N. bucera* provides additional evidence that the posterior lobes, which are attached to the supra-oesophageal ganglion of species such as *N. californiensis* and *N. caeca*, have been formed by the posterior migration of prostomial epidermal mucus-cells. In *N. picta* there are few mucus-cells in the prostomium. In *N. incisa* there are many, and some at the sides of the prostomium occur in clumps and project into the prostomial cavity. In *N. bucera* there are numerous mucus-cells, but they are massed at the sides of the prostomium and open to the exterior over a narrow zone. The cell-bodies are in intimate contact with the sides of the supra-oesophageal ganglion and lie within the membranes investing the brain. Subsequent evolution of the prostomial mucus-glands has led to the accumulation of their cell-bodies in the posterior part of the supra-oesophageal ganglion in the form of posterior lobes.

SOME species of *Nephtys*, particularly those from the north-east Pacific, but including the common circumpolar species *N. caeca*, have a pair of long lobes attached to the posterior margin of the supra-oesophageal ganglion. These lobes are filled with mucus-cells, the long necks of which run in a tract on each side of the ganglion and open to the exterior in the lateral walls of the prostomium by way of conspicuous lateral organs. The cells in the posterior lobes and the lateral tracts of cell processes are all enclosed within the sheath that invests the ganglion, and they and the lateral organs are separated from the ganglion-cells only by a layer of neuroglial tissue. In other species these lobes are missing and mucus-cells are confined to the lateral walls of the prostomium, more or less in the position occupied by the lateral organs in the former species, and have no connexion with the supra-oesophageal ganglion at all.

In all species of *Nephtys*, whether they have posterior lobes or not, there is a mass of mucus-cells in the middle of the anterior edge of the prostomium. These generally open to the exterior on the ventral surface, and there may be other mucus-cells in the lateral walls of the anterior part of the prostomium. However, in one species, *N. longosetosa*, while most of the mucus-cells in the medial group open to the exterior ventrally, and most of those in the lateral walls open to the side, a few cells in the medial group open to the side by way of long necks, forming a small lateral organ in the anterior part of the prostomium. In some other species, most obviously in *N. californiensis*, most of the cells in the medial group open to the exterior by way of long necks which

run to the lateral walls of the prostomium, forming a large anterior lateral organ on each side, which completely replaces the small mucus-cells in the epidermis at the sides of the prostomium, and which adjoins a similar posterior lateral organ associated with the posterior lobes. Anterior and posterior prostomial mucus-cell systems vary independently, and in *N. cornuta* there are well-developed anterior lateral organs, but no posterior lobes and, therefore, no posterior lateral organs.

When describing the mucus-gland system of *Nephtys* (Clark, 1955), I suggested that mucus-glands in the lateral walls of the anterior part of the prostomium had migrated into the medial mass, retaining their connexion with the lateral walls of the prostomium. *N. hombergi* is an example of a species with a complete separation of the lateral and medial mucus-cells, *N. longosetosa* represents an intermediate stage in which a few cells from the lateral walls have migrated into the medial group, and *N. californiensis* an extreme case in which all the mucus-cells opening to the lateral walls of the prostomium lie in the medial group. By analogy, I argued that the mucus-cells in the lateral walls of the posterior part of the prostomium must have hypertrophied and migrated back into the brain capsule, but retained their connexion with the prostomial walls so that they opened to the exterior by way of the lateral tracts and the lateral organs. The chief weakness in this interpretation lay in the absence of any intermediate steps in the process. The mucus-cells were found in the lateral prostomial walls or in the posterior lobes, but never half-way between the two in any species I had examined.

Recently I have had the opportunity of examining specimens of *N. bucera*, kindly sent to me by Dr. Marion Pettibone from various localities in Maine and New Hampshire, and I have re-examined specimens of *N. incisa* and *N. picta* from Massachusetts. This material has provided additional evidence about the evolution of posterior lobes in *Nephtys* and has provided the intermediate stages which were previously missing.

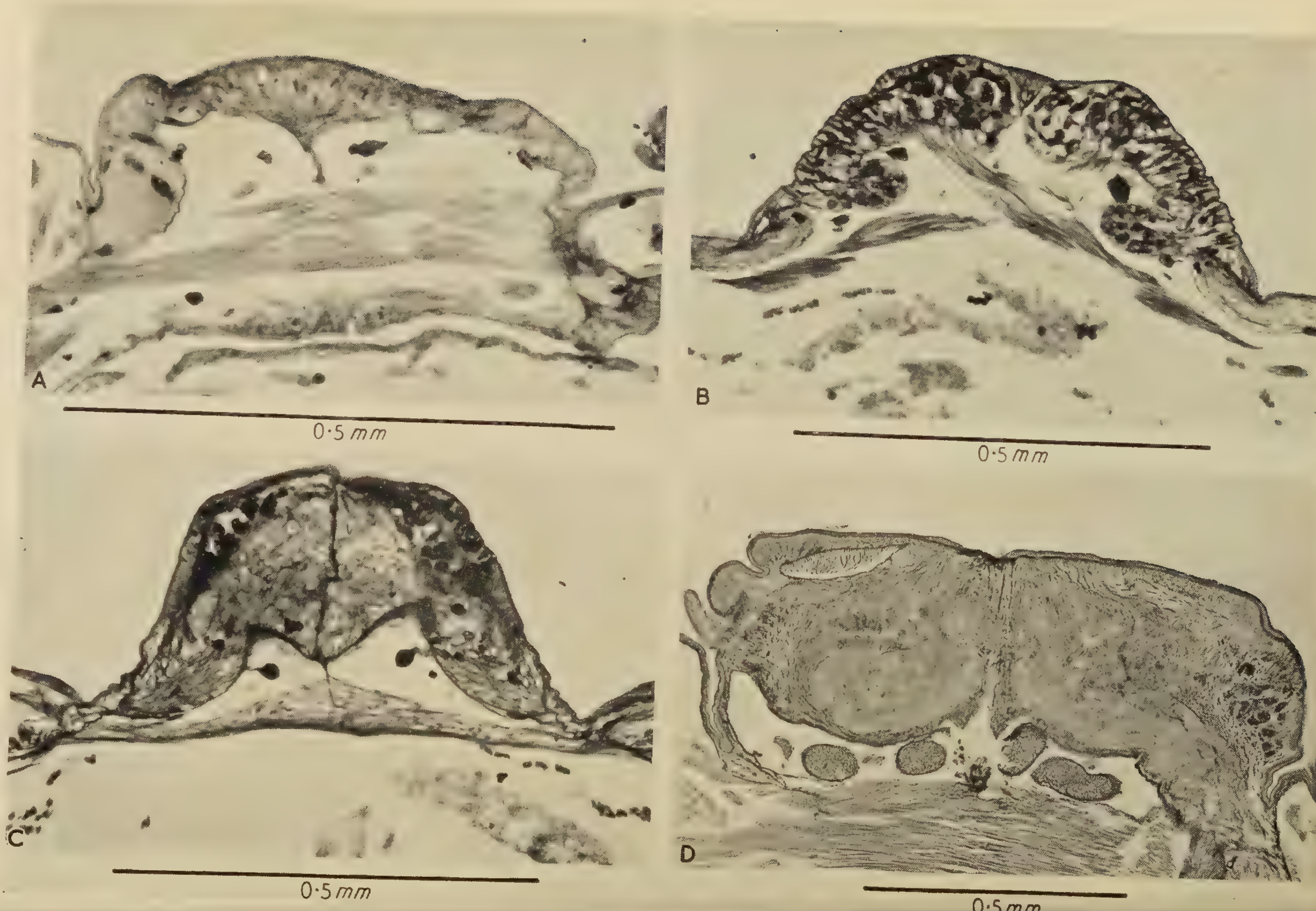
Material and Methods

Specimens of *N. bucera* Ehlers, *N. incisa* Malmgren, and *N. picta* Ehlers were fixed whole in Bouin's fluid. Frontal and transverse sections have been cut at 10μ and stained with paraldehyde fuchsin (Clark, 1955), Altmann's fuchsin and methyl green picrate (Gabe, 1947), Heidenhain's iron haematoxylin, or by Holmes's silver impregnation technique (Nicol, 1948).

Observations

None of the three species possesses posterior lobes, and the mucus-cells in the anterior medial group open to the exterior ventrally. There are thus no lateral organs and we are concerned solely with the arrangement of the mucus-cells in the lateral walls of the prostomium, particularly those in the posterior part of it that are in the neighbourhood of the supra-oesophageal ganglion.

The supra-oesophageal ganglion of *N. picta* lies mainly in the first body segment and only a small part of it is in the prostomium (fig. 1). The nuchal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organs, which mark the posterior limit of the prostomium, are at the level of the most anterior ganglion-cells in the brain. While the ganglion is attached to the dorsal surface of the prostomium, it makes no contact with the sides of it, although the circum-oesophageal connectives in front of the brain do so.

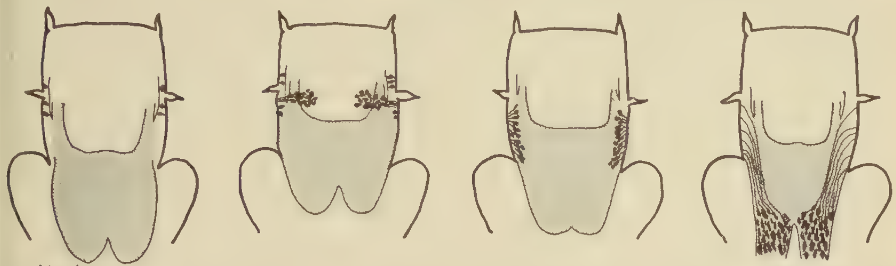

FIG. 1. Evolution of prostomial mucus-glands in the Nephtyidae. In *N. picta* there are only a few mucus-cells in the prostomial walls and these have no contact with the ganglion. In *N. incisa* there are more mucus-cells, and clumps of them project into the prostomial cavity. In *N. bucera* the mucus-cells are in contact with the ganglion, are enclosed within the ganglionic membranes, and are massed at the sides of the ganglion. In *N. caeca* there are far more mucus-cells, these have migrated into the posterior part of the ganglion but open to the exterior in the sides of the prostomium. Supra-oesophageal ganglion stippled, mucus-cells black.

They run in the sub-epidermal basement membrane. Compared with most other species of *Nephtys*, there are few mucus-cells in the prostomial epidermis and those that there are, are in the lateral walls and have no contact with any part of the nervous system (fig. 2, A).

The brain of *N. incisa* occupies a more anterior position than that of *N. picta* and most of it lies in the prostomium, so that the nuchal organs are near the back of the brain. As is the case in all species of *Nephtys* in which the brain is largely prostomial, it is attached not only to the dorsal part of the prostomium, but also to the sides of it. The circum-oesophageal connectives, as usual, run forwards and downwards from the brain in the sub-epidermal basement membrane of the prostomial walls. There are numerous mucus-cells in the prostomial walls (figs. 1; 2, C), most of which lie in the base of the epidermis and have narrow necks running directly to the cuticle through which they open by fine pores. There appear to be more and larger mucus-cells than can be accommodated in a single layer of epidermal cells, and a number of them are arranged in clumps at the sides of the prostomium and project into the prostomial cavity (fig. 2, B). These clumps of mucus-cells lie dorsal

FIG. 2 (plate). A, transverse section of the prostomium of *N. picta*, cut a little anterior to the supra-oesophageal ganglion. Bouin, paraldehyde fuchsin. B, transverse section of the prostomium of *N. incisa*, cut a little anterior to the supra-oesophageal ganglion, showing a clump of mucus-cells projecting from the epidermis into the prostomial cavity on each side. Bouin, paraldehyde fuchsin. C, transverse section of the prostomium of *N. incisa*, including the anterior part of the supra-oesophageal ganglion. Numerous dark-staining mucus-cells occur in the epidermis. Bouin, paraldehyde fuchsin. D, slightly oblique transverse section of the prostomium of *N. bucera*, showing massed mucus-cells at the right-hand side of the supra-oesophageal ganglion. Bouin, paraldehyde fuchsin.

to the circum-oesophageal connectives and occur only anterior to the supra-oesophageal ganglion, so that they do not form any connexion with the nervous system.

The walls of the prostomium of *N. bucera* are curious in that a thickened ridge of epidermis runs along the middle of each side and the prostomial mucus-cells are concentrated in it almost to the exclusion of other epidermal elements. The mucus-cells are large and have long, narrow, and slightly coiled necks. The cuticle along the line of the two ridges is peppered with the pores through which these mucus-cells open to the exterior. Massed mucus-cells occur at the sides of the prostomium from the level of the posterior antennae almost to the nuchal organs. The supra-oesophageal ganglion of *N. bucera*, like that of *N. incisa*, occupies a relatively anterior position and is largely prostomial. It extends from side to side of the prostomium and is in intimate contact with some of the prostomial mucus-glands (figs. 1; 2 D). The latter extend anterior to the brain and lie dorsal to the circum-oesophageal connectives, but in the posterior part of the prostomium they lie within the membranes investing the ganglion (extensions of the sub-epidermal basement membrane) and are separated from the lateral groups of nerve-cells only by a barrier of neuroglial fibres. Some of these penetrate between the mucus-cells and there appears also to be a number of connective tissue elements in the zone between the ganglion proper and the cuticle; ordinary, structural epidermal cells are missing from this region of the prostomium.

Discussion

The disposition and form of the mucus-cells in the prostomium of *N. incisa* and *N. bucera* suggest how posterior lobes may have evolved from purely epidermal mucus-cells. The function of the mucus is unknown, but evidently there is a need for it to be released in copious quantities at the sides of the prostomium in some species. *N. picta* is exceptional because it has very few mucus-cells in the prostomium, but in other species there is either a considerable number of them in the prostomial walls or else they fill the posterior lobes and open to the exterior by way of the lateral organs at the sides of the prostomium. An incipient hypertrophy of the prostomial mucus-gland system may be seen in *N. incisa*, in which mucus-cells are particularly numerous in the entire prostomial epidermis and, further, groups of them bulge inwards into the prostomial cavity, so permitting a greater number of mucus-cells than can be accommodated in the epidermis to open to the exterior. In *N. bucera* the arrangement of the prostomial mucus-cells is more specialized. These cells are restricted to ridges at the sides of the prostomium and there are relatively few elsewhere on the prostomial epidermis. The cells have long, narrow necks and so, although there are many of them, they open to the exterior over a narrow zone running along the sides of the prostomium.

This concentration of mucus-cells brings them inevitably into contact with the supra-oesophageal ganglion. The brain of *Nephtys* is epidermal (Clark, 1958), as are the mucus-cells, and in the posterior half of the prostomium it

replaces the ordinary epidermal cells. There is thus no ganglionic sheath which, in *N. bucera*, might separate the lateral mucus-cells from the ganglion; both are invested by the sub-epidermal basement membrane and the mucus-cells are separated from the nerve-cells only by neuroglial tissue. Once this stage of organization has been reached, further development of the mucus-gland system must be influenced by the presence of the ganglion. The only way in which a substantial increase in the number and size of the mucus-cells can be achieved is by their migration in a posterior direction so that they can expand into the body cavity, retaining their connexion with the prostomial walls by long ducts. In *N. incisa*, to be sure, the mucus-cells have extended into the prostomial cavity, but there is space for only very limited development in that direction. In all the species that have a highly developed mucus-gland system, the extension of these cells has been into the body cavity. The mucus-cells of *N. bucera* have long, coiled necks and those of the more posterior cells run forwards, suggesting that even in this species there has been some posterior migration of the cell-bodies. A further migration in a posterior direction must inevitably result in the cells eventually lying in the posterior part of the ganglion (see fig. 1, p. 507). This is the situation in *N. californiensis* and *N. caeca*, and in *N. californiensis* there are a few mucus-cells occupying a lateral position in the lateral tract of ducts that runs from the cell-bodies in the posterior lobes to the lateral walls of the prostomium (Clark, 1955).

No further information about the function of the mucus-gland system has been discovered since the original description of the posterior lobes. The factors which must be taken into account in any explanation of their function are now clearer. In *N. picta*, a species which appears to be primitive in several respects (Clark, 1957), there are virtually no prostomial mucus-glands. In *N. incisa* there are many, but they are dispersed over the whole prostomial epidermis though the larger clumps of glands open laterally. In *N. bucera* the mucus-cells are concentrated and open in a narrow zone along the sides of the prostomium. In *N. californiensis*, *N. caeca*, and many other species, the mucus-gland system is enormously developed but the cells still open to the exterior along the sides of the prostomium, and in a few species (e.g. *N. californiensis* and *N. caecoides*) this copious supply of mucus at the sides of the prostomium is supplemented by the discharge from mucus-cells in the anterior median part of the prostomium. In *N. cirrosa* the mucus-cells of the posterior lobes appear to have been highly modified and reduced in number, and it may be supposed that in this species they serve a different function.

I am grateful to Mr. K. J. Wood for taking the photomicrographs.

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The Cultivation in the Living Organism of the Thymus Epithelium of the Guinea-Pig and Rat

By CH. GRÉGOIRE

(*From Institut Léon Fredericq, University of Liège, Belgium*)

With one plate (fig. 1)

Summary

In autografts of thymus enclosed in porous cellulose membranes, the epithelium grows out in the shape of nodules, compact sheets, cords, and scattered isolated islands. During the experimental period, regeneration of the thymocytes did not take place in the grafts, provided that exogenous lymphocytes from other parts of the body were prevented by the cellulose barrier from penetrating the remnants of the thymic epithelium.

Introduction

In autografts and in syngenesiografts of the thymus, immigration of lymphocytes into the epithelial remnants of the transplant makes an important contribution to the regeneration of the organ (Jolly and Lieure, 1932; Grégoire, 1935, 1939; Law, 1952).

In previous investigations on the reciprocal reactions of the thymocytes and of the epithelial reticulum (Grégoire, 1935), I tried to prevent this immigration by isolating the grafts inside filters that allowed the passage of metabolites but were impermeable to infiltrating cells. Fragments of lobules from normal and irradiated, adult and embryonic thymuses were enclosed in collodion bags (Rezzesi's procedure, 1932) or in peritoneum impregnated with metallic silver (Irwin, Gairns, and Banting's procedure, 1934), or were wrapped in filter paper and in hydrophil cotton sheets. The loaded containers were implanted subcutaneously or intraperitoneally.

Proliferation of the thymic epithelium was recorded, especially in autologous and in embryonic grafts wrapped in cotton envelopes and temporarily protected by these envelopes from lymphovascular connexions and from infiltration of migrating cells and of lymphocytes (Grégoire, 1935, plate XIX, figs. 15, 16, and 39, and text-fig. 12). However, the cotton procedure was rather unsatisfactory, owing to the unknown variations in the weave of the cotton material, and owing to the difficulty of detecting the thinly spread epithelial structures growing inside the granulome with giant cells developed around the cotton fibres.

In further investigations, reported in the present paper, advantage has been taken of the improved methods of manufacturing cellulose membranes of standard grades of porosity.

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Material and Methods

Thirty guinea-pigs, $2\frac{1}{2}$ to 3 months old, were used for the autografts, and 20 rats, 6 weeks old, for the homografts. Approximately 120 transplants were studied. In the guinea-pigs, total thymectomy was performed during the operation, and the grafts were the only thymic tissue present during the experimental period.

After excision of the parathyroid gland included in the thymus, the interlobular connective tissue was trimmed away and the thymic lobules were cut into small fragments. These were placed in the centre of the porous membranes which were rolled or folded around the pieces of tissue. In order to insure a close contact between transplants and membranes, the folds were held tight by means of catgut knots or silver clamps. The bags were not as completely closed as if they had been sealed, but several folds separated the grafts from the surrounding fluids and penetration of free migrating cells into the bags was sufficiently delayed and was not noticeable during the experimental period. The loaded bags were placed in the subcutaneous connective tissue or in the peritoneal cavity. The 'diffusion chamber technique' recently developed (Prehn, Weaver, and Algire, 1954) has also been used in the present study.

Some of the porous cellulose membranes used were prepared according to the methods initially elaborated by Elford (Elford and Ferry, 1934). Others were 'dry filter membranes of Zsigmondy', of graded porosity, manufactured by the Membranfiltergesellschaft, Sartoriuswerke, Göttingen, Germany. The diameter of the pores of these membranes is 1–3 μ for the coarse grade, 0·5–1 μ for the medium grade, and 0·2–0·5 μ for the fine grade.

The membranes with their content were removed from the guinea-pigs between the 6th and 29th day, and from the rats on the 16th day after the operation. They were fixed in Bouin–Hollande–sublimate or in Susa, embedded in paraffin, cut in semi-serial sections (5 μ), and stained with Masson's trichrome and May–Grünwald's stains.

Results

During the days following transplantation, the debris of the massive destruction of the thymocytes remained in place, underwent slow dissolution, or was resorbed by epithelial and mesenchymatous phagocytes. The epithelial reticulum, initially loose, shrank into a denser stroma, in which the large characteristic mitoses of its cells appeared and increased in number with time (fig. 1, c). The epithelial remnants of the grafts grew out and formed nodules and compact sheets (fig. 1, A–E), cords (fig. 1, G), or small scattered islands consisting of a few cells. In some areas the epithelial cells were stretched and spindle-shaped. In these areas it was sometimes difficult to distinguish these elongated elements from the mesenchymatous cells mixed with them.

A luxuriant epithelial proliferation occurred in the places where the epithelial remnants were in close contact with the inner surface of the porous

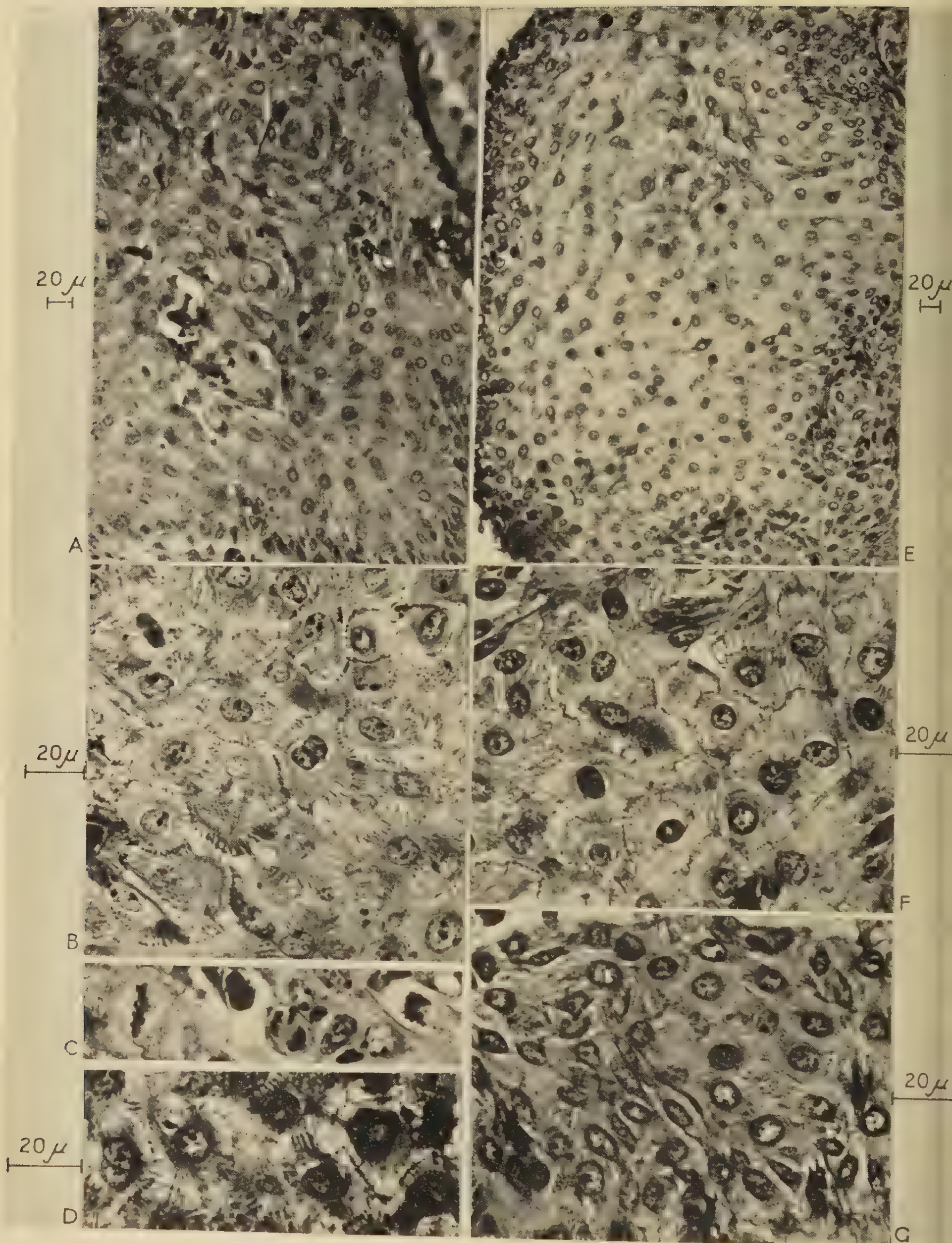

FIG. 1

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membranes (fig. 1, A–E). In the central parts of the bags, the outgrowth of the thymic epithelium was less active. Floating fragments, in too loose bags, were necrotic.

In the sections of the nodules and in various parts of the cords (fig. 1, B, D, F, G) the epithelial tissue looked like a flagstone pavement in which the individual cells assumed the shape of flat polygons, connected together by intercellular bridges, on which knots of Bizzozero-Ranvier were visible (fig. 1, D).

Thymocytes were absent in the sheets of the actively growing epithelium. In some portions of the epithelial remnants corresponding topographically to the former medullary zone of the lobules, a few thymocytes, with a darkly staining nucleus, had escaped the massive disintegration at the beginning of transplantation, and were scattered between the epithelial cords. These cells had obviously not increased in number during the experimental period, which extended, in several grafts, up to 29 days after transplantation.

The appearance of the grafts enclosed in porous membranes contrasted strikingly with that of the free grafts after the same period of time. In these free grafts, the three successive phases of regeneration of the thymocytes—(1) the immigration of exogenous small lymphocytes, with a darkly staining nucleus, into the epithelial remnants of the grafts; (2) development of a transient lymphoblast phase; and (3) the active mitotic proliferation and transformation of these blasts into small thymocytes with faintly staining nuclei (Grégoire, 1935, 1939)—resulted in reconstitution of the normal appearance of the lobules, in about 10 to 15 days. In the reorganized lobules, swarming thymocytes concealed the cells of the epithelium.

Sixteen days after transplantation, the portions of the homologous grafts in contact with the porous membranes had survived. The histological features of these grafts were similar to those described above for autografts.

FIG. 1 (plate). Guinea-pigs, 100 days old. Autografts of thymus, enclosed in collodion bags (diameter of the pores, 200–500 mμ) placed for 22 days in the subcutaneous connective tissue. A–E, portions of epithelial nodules spread out along the membranes in close contact with them. In A, a part of the membrane is shown at the top right side of the picture. In E, the membrane has been detached from the graft during the sectioning process (top and bottom left). Notice, in both pictures, the mosaic-like architecture of the cells, an arrangement common to all epithelial cells in cultures *in vitro*. A, the epithelial cells are elongated and disposed in palisades along the boundaries of the nodule. E, in the central part of that nodule, the epithelial cells are spread out (pale cytoplasm). In the peripheral regions of the same nodule, the epithelial tissue is denser and the cells are grouped in strands and in cords, in continuity with other epithelial structures scattered in a loose mesenchymatous tissue in the more central portions of the bag. Hassall's corpuscles (not included in the pictures A–E) are located inside the epithelial outgrowth. B, D, F show portions of nodules more highly magnified. Individual epithelial cells appear in section like a pavement of polygonal flagstones, and are connected together by intercellular bridges (with knots of Bizzozero-Ranvier on their middle part), disposed all around their boundaries. Granules gathered in small fields (B–D) correspond to intercellular bridges in transverse section. In B, a binucleated cell appears in the centre of the picture. C shows three epithelial mitoses (2 metaphases, 1 anaphase) in a cord such as that illustrated in G. G shows a section of cords visible in the right part of E. In the central part of the cords the cells are polygonal. At the periphery they assume an elongated fusiform aspect. Mesenchymatous cells in small numbers are scattered between the cords.

Discussion

In the present material, survival and proliferation of the epithelium was recorded in membranes of the three porosities used. The grade of porosity seemed to play a less important part than the adherence of the fragments to the cellulose membranes.

Enclosed in porous membranes during the life of the animal, the thymic epithelium assumes the characteristic mosaic-like architecture recorded in all epithelial tissues *in vitro* (Fischer, 1946). Its outgrowth does not essentially differ from that described in cultures *in vitro*, especially by Pappenheimer (1913), Wassén (1915), Tschassownikow (1926), Popoff (1927), Schopper (1934), Emmart (1936), and Murray (1947).

Intercellular bridges, of controversial significance (Pease, 1951; Medawar, 1953), have been observed in autografts of thymus by Tschassownikow (1926) and by Jolly and Lieure (1932), and in thymic epithelial sheets growing inside cotton envelopes (Grégoire, 1935).

According to Algire (1957), membranes with pore diameters of 0·8μ allow the passage of migrating cells. Owing to the relatively short duration of the present experiments, infiltration by migrating cells through membranes corresponding to those used by Algire was not recorded.

In the transplants of thymus enclosed in porous membranes, the only sources available for the proliferation of thymus lymphocytes and for the re-population of the epithelial stroma, are either the rare small cells mentioned in the description (homoplastic lymphocytopoiesis) that escaped the initial massive disintegration, or the mesenchymatous elements blended with the epithelial components of the grafts (heteroplastic lymphocytopoiesis).

An inadequate supply of metabolites essential for the multiplication or neoformation of these lymphocytes seems to be unlikely, especially in the vicinity of the porous membranes, where mitoses were observed in the connective cells as in the epithelial cells.

The present results rather support my former conclusions (Grégoire, 1935) that, as long as the lymphocytes cannot reach the epithelial cells of the thymus transplanted during life, the transplant remains purely epithelial. The results are also consistent with previous observations that immigration of exogenous lymphocytes, with duplication of the histogenetic process, plays an important part in the regeneration of the experimentally involuted thymus, especially in grafts (Jolly and Lieure, 1932: Grégoire, 1935, 1939; Law, 1952), and that heteroplastic lymphocytopoiesis is weakly developed in the thymus (see discussion in Grégoire, 1935, 1939, 1945, 1956; Downey, 1948; Frank, Kumagai, and Dougherty, 1953; Santisteban and Dougherty, 1954).

In former studies on the influence of endocrine imbalance on the regeneration of the irradiated thymus, alterations of the epithelial cells were recorded after adrenalectomy (Grégoire, 1942) and after injection of testosterone propionate (Grégoire, 1945). The procedure of culture during the life of the animal in porous membranes reported in this paper has been found to be an

adequate technique for reinvestigating these reactions in the absence of the lymphoid components of the organ. The results will be reported later.

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The Cytoplasmic Inclusions of *Nyctotherus macropharyngeus*: Histochemical Studies

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Summary

The cytoplasm of *Nyctotherus macropharyngeus* contains lipid bodies, mitochondria, and carbohydrate bodies. The lipid bodies consist of neutral lipids, most probably triglycerides, and protein. The mitochondria are rod-shaped. The carbohydrate bodies are formed by the coalescence of smaller spheres, which appear to arise in close association with the mitochondria. The external part of the smaller spheres is PAS-positive.

Introduction

RICHARDSON and Horning (1931) described polymorphic 'Golgi bodies' in *N. cordiformis*, varying in shape from short rods to twisted filaments. In the same species Patten (1932) described certain twisted, snake-like objects as Golgi bodies. Khajuria (1950) gave the same name to granules in the cytoplasm of *N. macropharyngeus*.

Horning (1927) and Richardson and Horning (1931) described the mitochondria of *N. cordiformis* as rod-shaped; Patten (1932) described them as spherical granules. Khajuria (1950) described those of *N. macropharyngeus* as globular and rod-shaped.

'Vegetative granules' have been described in *Nyctotherus* by the authors named above. They appear as transparent spheres in the living organism, and are unstained by iron haematoxylin in fixed preparations. In the present paper they will be called carbohydrate bodies.

As far as the author is aware, no paper dealing with the histochemistry of the cytoplasmic inclusions of *Nyctotherus* is available. The purpose of the present work was to investigate, both morphologically and histochemically, the cytoplasmic inclusions of *N. macropharyngeus*.

Material and Methods

Living specimens were examined in 0·85% sodium chloride solution by phase-contrast microscopy. Janus green B, neutral red, and other vital dyes were also used.

Specimens were placed in 2% osmium tetroxide solution and examined without further treatment.

The following fixatives were used for specimens that were to be sectioned subsequently: Lewitsky (strong Flemming without acetic), Regaud, Helly, Bouin, Carnoy. Paraffin sections were cut at 5μ. They were stained either with iron haematoxylin (0·5% haematoxylin) or with acid fuchsine (Cain, 1948*b*).

Details of the histochemical tests used are given in the appendix (p. 521).

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Observations

In addition to the contractile vacuoles and food vacuoles, there are thre[e] distinct types of cytoplasmic inclusions: lipid bodies, mitochondria, an[d] carbohydrate bodies.

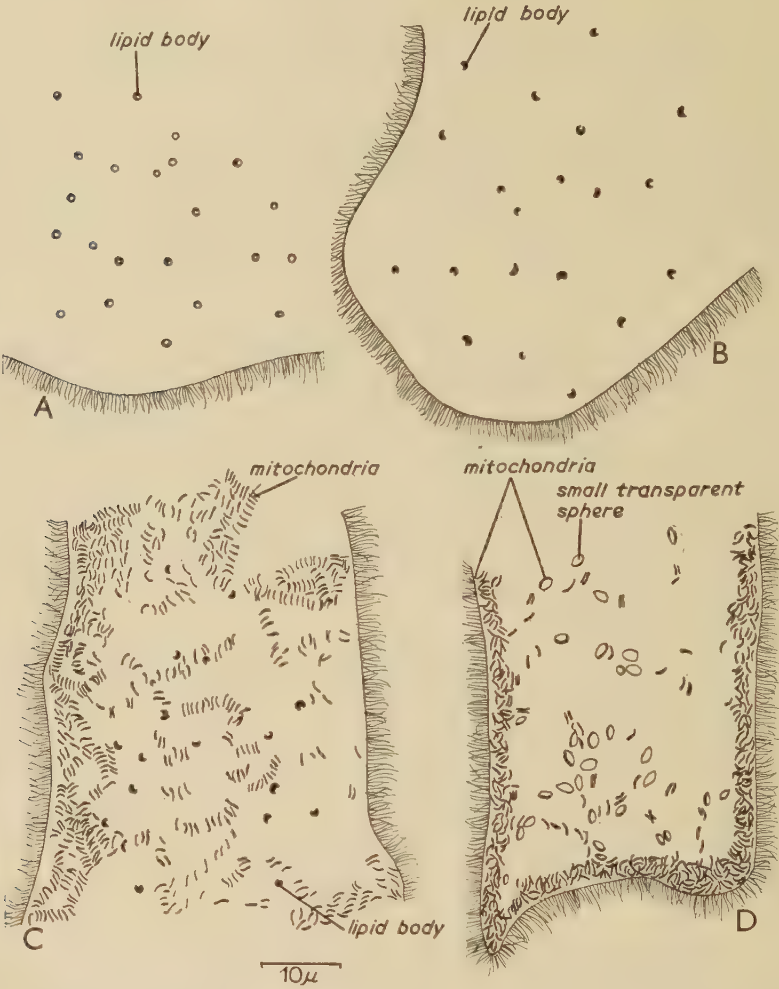

Fig. 1. Camera lucida drawings of portions of *N. macropharyngeus*. A, fresh material treated with 2% osmium tetroxide. B, formaldehyde-calcium, postchromed, Sudan black at 60° C. C, weak Bouin, pyridine extraction, mercuric bromophenol blue. D, Helly, Cain's acid fuchsine.

Lipid bodies. These are distributed at random in the medulla. Each appears to be duplex, consisting of externum and internum. The duplex structure is seen in the living organism and also after treatment with osmium tetroxide solution (fig. 1, A) and with Sudan black (fig. 1, B). The externum is darkened by the two latter techniques, while the internum remains clear. The externum appears crescentic in Sudan black preparations, and also in preparations

coloured by mercuric bromophenol blue (fig. 1, C). The latter result suggests the presence of protein in the externum. Ciaccio's 'unmasking' technique (see Gupta, 1958), followed by colouring with Sudan black, gives the same crescentic appearance as is seen in ordinary Sudan black preparations. The internum evidently contains no lipid; indeed, it did not give a positive response to any of the histochemical tests that were tried.

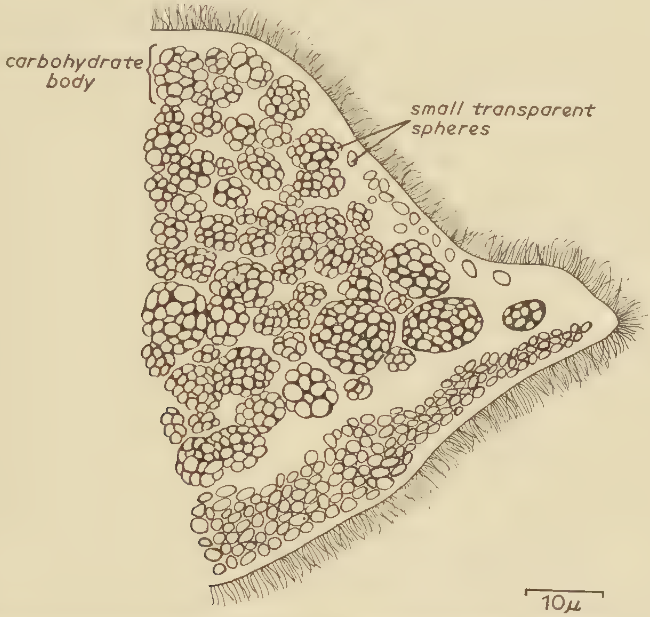

FIG. 2. Camera lucida drawing of a portion of *N. macropharyngeus*. Bouin, PAS. Only the outer parts of the small spheres are PAS-positive.

Mitochondria. These are rod-shaped. They are arranged in a peripheral layer just below the pellicle, and some are scattered also in the medulla. They are stained by mercuric bromophenol blue (fig. 1, C) and by acid fuchsine (fig. 1, D); they are also darkly stained by iron haematoxylin in material fixed in Lewitsky, Regaud, or Helly. They stain supervitally with Janus green.

Carbohydrate bodies. Each of these is composed of numerous small spheres (fig. 2). The superficial part of each sphere is PAS-positive. The carbohydrate bodies give no positive response to tests for proteins or lipids, and are not stainable by neutral red during life.

The small spheres develop in close association with mitochondria (fig. 1, D). As each sphere grows, the associated mitochondrion begins to lose its affinity for acid fuchsine. Ultimately, a number of spheres coalesce to form larger bodies.

DISCUSSION

Lipid bodies. The evidence suggests that the externum consists of neutral lipid, probably triglyceride, and protein. The composition of the internum is unknown.

In *Opalina ranarum* the lipid bodies consist of triglyceride and lipoprotein; in *O. scalpriformis*, of lipoprotein only (Dutta, 1958). These two species and *N. macropharyngeus* live in the same environment, as gut parasites of frogs and toads, but their lipid bodies differ considerably in chemical composition.

The lipid bodies correspond to the so-called 'Golgi bodies' of earlier workers (Richardson and Horning, 1931; Patten, 1932; Khajuria, 1950).

The carbohydrate bodies or 'vegetative granules' were supposed by Horning (1927), Richardson and Horning (1931), and Khajuria (1950) to function as nutritional stores. It is possible that they develop under the influence of enzymes produced by mitochondria, as suggested by these authors.

This work was carried out in the Department of Zoology, Panjab University, Hoshiarpur, under the supervision of Prof. Vishwa Nath, Head of the Department, to whom I am greatly indebted for allowing me the necessary laboratory facilities. I am thankful to Mr. Brij L. Gupta, technician to the Department, for some useful help and suggestions.

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Technique	Fixation	Embedding medium	Thickness of sections	Reference	Lipid bodies	Mito-chondria	Carbo-hydrate bodies
SB in 70% ethanol	FCa and FCa+PC	G	5 μ	Baker, 1946, 1956	++c	—	—
SB in 70% ethanol at 60° C.	,,	,,	,,		++c	—	—
SB in propylene glycol	,,	,,	,,	Chiffelle and Putt, 1951	++c	—	—
SB* cold acetone	Fresh or FCa	,,	,,	Krishna, 1950	—	—	—
SB* cold ether	,,	,,	,,	Pearse, 1954	—	—	—
SB* cold ethanol	,,	,,	,,	,,		—	—
Sudan III and IV in 70% ethanol / acetone	FCa and FCa+PC	,,	,,	Kay and Whitehead, 1941	++c	—	—
Fettrot in 70% ethanol	,,	,,	,,	Pearse, 1954	++c	—	—
Pink, Nile blue	,,	,,	,,	Cain, 1947, 1948*a*	pink, ++c		—
Nile blue* cold acetone	Fresh or FCa	,,	,,	,,			—
AH	FCa+PC	,,	,,	Baker, 1946		+	
AH*PE	WB and PE	,,	,,	,,	—	+	
Fischler's reaction	FCa and FCa+PC	,,	,,	Pearse, 1954	—	—	
Feyrter's enclosure	FCa+PC	,,	,,	,,	blue		
PAS	B, C, H, &c.	P	,,	Hotchkiss, 1948; Pearse, 1954	—	—	+++
PAS* acetylation	,,	,,	,,	McManus and Cason, 1950	—	—	
PAS* O.IN.KOH	,,	,,	,,	McManus and Cason, 1950	—	—	+++
Performic acid / Schiff	FCa	G	,,	Pearse, 1954; Lillie, 1952	++c	—	++
MBB	FCa, FCa+PC, B, C, &c	G or P	,,	Mazia and others, 1953	++c	+++	—
MBB*PE	WB and PE	G	,,	,, ,,	++c	+++	—
Cholesterol reactions	FCa	,,	,,	Schultz, 1924, 1925; Pearse, 1954; Gomori, 1952; Romieu, 1927	—	—	—
Ciaccio's technique	FCa and phenol	,,	,,	Bradbury, 1956	++c	—	
2% osmium tetroxide	Fresh			Nath, 1957	++r	—	—

Key: AH = acid haematein; B = Bouin; C = Carnoy; c = 'crescent'; FCa = formaldehyde-calcium; G = gelatine; H = Helly; MBB = mercuric bromophenol blue; P = paraffin; PAS = periodic acid / Schiff; PC = with post-chroming; PE = pyridine extraction; r = rings; SB = Sudan black B; WB and PE = weak Bouin followed by pyridine extraction; + = weak reaction; ++ = moderate reaction; +++ = strong reaction; — = negative; * = after treatment with.

The Fine Structure of the Mesenteries of the Sea-Anemone *Metridium senile*

By A. V. GRIMSTONE, R. W. HORNE, C. F. A. PANTIN, *and* ELAINE A. ROBSON

(*From the Zoological Laboratory, Cambridge*)

With six plates (figs. 2–7)

Summary

Sections of the retractor and radial face of mesenteries of *Metridium senile* (L.) fixed with osmic, osmic-phosphotungstic, or formol-phosphotungstic fixatives were examined by electron microscopy. Each muscle-fibre forms the basal part of a musculo-epithelial cell and is in contact through the cell membrane with a differentiated surface layer of the underlying mesogloea. The muscle-fibres bear crests of cytoplasm containing mitochondria. Each crest is continuous with a cytoplasmic stem which passes through the intercellular space into the epithelial part of the musculo-epithelial cell. The muscle-fibre consists of densely packed longitudinal filaments 40–80 Å across. The muscle-fibres present a similar appearance over a great range of extension of the muscle. The filaments show no gross periodic structure like that of striated muscle; nor has any fine regular structure like that described in some other 'plain' muscle-fibres yet been detected. There is, however, an irregular beading on the filaments at about 240 Å. Though much smaller, the muscle-fibres of the very slowly contracting radial muscle sheet have the same fine structure as those of the very rapidly contracting retractor muscle. The epithelial part of the cell bears a flagellum arising from a cytoplasmic 'crater'. The flagellum contains vesicles as well as the typical fibrils. It passes continuously into a complex basal corpuscle which continues into a striated fibrous root. Near the epithelial surface there are conspicuous cross-connexions between adjacent cells. Mucus cells and amoebocytes are described. The mesogloeal lattice of Chapman, which permits free extension of the actinian body, is composed of fibres which seem to be built up of tubular fibrils of about 100 Å diameter. The fibrils show well-developed banding at 260 Å. Where fibrils adhere together their banding is congruent. The criteria of correspondence between the electron micrographs and the living structure are discussed, and the value of information from artifacts and varied methods of fixation is noted.

Introduction

COMPARED with the higher animals, the Actinozoa are very simply organized. The body is built up of sheets of tissue, on each surface of which there is an epithelium, resting upon a middle layer of fibrous mesogloea. This generally contains no cellular elements other than some scattered amoebocytes and, locally, genital cells.

The structure of the mesenteries of *Metridium senile* (L.) is typical of this organization. The fibrous mesogloea separates two epithelia, in this case both endodermal. At its surface of contact with the mesogloea each epithelium bears a sheet of muscle-fibres consisting of a single layer. This muscle-sheet may be folded to produce muscles of considerable size, but even when this is so it

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remains essentially two-dimensional, and but one fibre thick. On one surface of the mesentery the fibres run vertically along the oral axis and form the retractor muscle, on the other surface the fibres run transversely (fig. 1, A).

Batham and Pantin (1950 *a* and *b*, 1951) have given an account of the structure and the mechanics of action of these muscle-sheets. Physiologically they are of particular interest because of their great contractility, to less than one-fifth of their extended length, the absence of any fixed resting length between

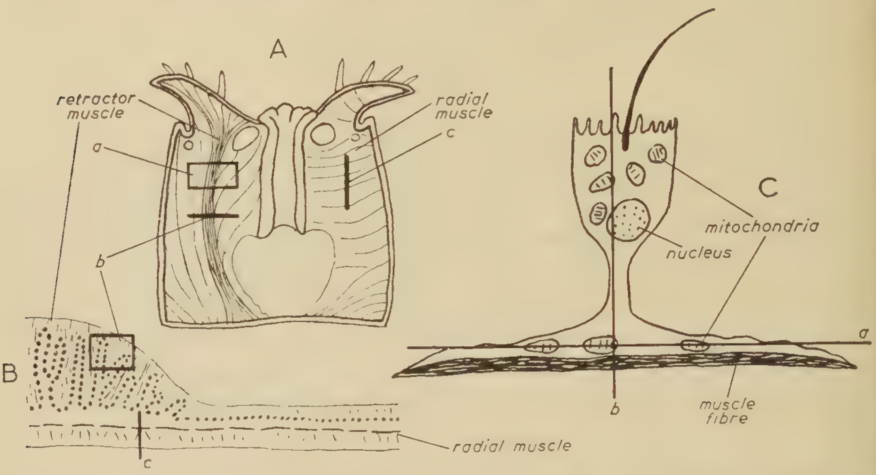

FIG. 1. Diagram to show plane of sections *a*, *b*, *c*. A, section through whole *Metridium* exposing two 'perfect' mesenteries. Left shows a retractor muscle surface, right shows a radial muscle surface of mesentery. B, transverse section of part of a 'perfect' mesentery showing retractor above and radial muscle beneath. G, stylized epithelio-muscular cell of mesentery showing basal muscle-fibre with cytoplasmic crest and mitochondria above; and stem connecting this with flagellate epithelial part of the cell.

these limits, and because of the great tension per unit area of cross-section they seem able to exert. All the muscle-sheets can contract slowly, taking a minute or more to develop tension. Under different conditions of excitation some of them can also contract rapidly, developing tension in a fraction of a second.

Robson (1957) has shown that each muscle-fibre of these sheets is the basal part of a musculo-epithelial cell. The superficial parts of these cells form a flagellate pavement epithelium; each epithelial part being connected with its muscle-fibre by a strand of cytoplasm which fans out as a crest along the fibre. Between these strands is a sub-epithelial fluid of considerable mechanical importance.

The mechanical properties of the muscle-fibres must be related to their fine structure. Since their diameter is of the order 0·5 to 1·0 μ it is not possible to see this with the light microscope, though it is well suited to the electron microscope. Likewise, the fine structure of the epithelial parts of the cells and of their connecting strands can be investigated only by this means.

Of the structure of the mesogloea to which the muscle-sheets are attached

good deal is now known. Chapman (1953) working with the light microscope owed that the mesogloea is organized in actinozoans as a three-dimensional ttice which permits great reversible extensibility. The lattice consists of colgen fibres in a fluid medium. The fibres tend to run more or less at 45° to e main axes of the animal, and to be arranged in alternating layers running bliquely right and obliquely left. The layers of the lattice are not wholly parate, and individual fibres may be seen to pass from one layer to the her.

Chapman raises the important question of whether this lattice structure uld arise by mechanical stresses set up in a matrix of fibrous material, by the ovements of the animal. He produces interesting arguments by analogy in pport of that. Here again interpretation of the organization must depend on knowledge of fine structure of the collagen fibres of the mesogloea; and ese, seeming to be between 0·1 and 1·0μ, are near the limits of resolution of e light microscope.

Though the actinozoan mesogloea is extensible, it must provide a support r muscle-fibres of the muscle-sheets. Batham and Pantin (1951) showed that e shortening caused by contraction of one muscle-sheet was accommodated buckling of the opposing muscle-sheet on the other surface of the mesooea. This sheet was thus passively thrown into folds at right angles to the rection of contraction. They argued from this that the part of the mesogloea immediate contact with the muscle-layer must have a different and less tensible character from the mass of the mesogloea. There is some histogical evidence for the existence of such a differentiated layer. But here again e structure cannot be fully analysed by the light microscope.

The objects of the present paper are thus to throw light on the fine structure the muscle-fibres and the cells of which they are a part, and to see how far e fine structure of the fibrous mesogloea will account for its special prorties.

Methods

Moderately large *M. senile* were obtained from the Marine Biological boratory, Plymouth. The typical procedure was as follows. The *Metridium* s anaesthetized for about 3 to 5 h in sea-water with an equal part of isotonic gCl_2 (0·36 M). These animals can recover perfectly after exposure to this xture for as long as 24 h. The anemone was cut open and a small wax plate s slipped under one of the 'perfect' mesenteries with the retractor face uprds. To avoid distortion the mesentery was pinned round its margin to the x plate. The preparation on the plate was then cut free from the animal and opped into fixative.

Three methods of fixation were used:

(1) Some specimens were fixed in ice-cold 1% OsO_4 in sea-water for 30 min. They were then washed in ice-cold sea-water for 15 min. At this stage, small pieces, about 0·5 mm square or less, of the fixed mesentery were cut out with razor blades under a binocular microscope.

(2) Some specimens were fixed in 1% OsO_4 and washed in sea-water as described above, and then small pieces were transferred to ice-cold 1% phosphotungstic acid in sea-water for about 30 h. They were then washed in ice-cold sea-water for 3 min (see Hanson, 1957).

(3) Some specimens were fixed for about 30 h in ice-cold neutral formalin, made up with sea-water to about 5% CH_2O. Pieces were cut out, washed in sea-water, passed to 1% phosphotungstic acid for 24 h, and washed as before.

Material prepared by each of these methods was dehydrated by passing through ice-cold 50%, 70%, 85%, and 95% ethanol, allowed to warm up to room temperature in the last of these, and then transferred to absolute ethanol at room temperature. Specimens were then passed through several changes of a mixture of butyl and methyl methacrylates (9 : 1 or 3 : 1) and finally into pre-polymerized methacrylate containing 1% benzoyl peroxide. Polymerization was at about 60° C. Flat embedding (Borysko, 1956) was commonly used. Sections were cut on a microtome of the type described by Hodge, Huxley, and Spiro (1954), floated on 25% acetone, and picked up on collodion-coated grids. They were examined without removing the embedding medium in a Siemens electron microscope (Elmiskop I). Orientation was sometimes additionally checked by observation of sections under phase contrast by the light microscope.

The osmic-phosphotungstic preparations gave by far the clearest detail. The fixation with OsO_4 alone was good, but the contrast was weak, particularly in the mesogloea. The formalin-phosphotungstic preparations showed excellent contrast but manifestly imperfect fixation of some fine structures. Useful information was, however, gained by comparison of sections prepared by the three methods. The criteria of good fixation will be discussed later.

Results

Retractor muscle-fibres and cytoplasm

The muscle-fibre is the evidently contractile part of the musculo-epithelial cell. It consists of a single strand attached to the mesogloea. With the light microscope the retractor muscle can be seen to bear a crest of cytoplasm. The crest passes into a narrow stem running to the epithelial part of the cell, which bears a flagellum (fig. 1, c). Between the stems is the intercellular fluid; the 'subepithelial fluid' of Robson. All the cytoplasm contains mitochondria; and these can be stained with Janus green B (Robson, 1957).

Under the light microscope, no essential difference can be seen in the structure of the muscle-fibres from different parts of the actinozoan body, except in size (Batham and Pantin, 1951). In the mesentery, the rapidly acting retractor muscle-fibres are about 1 mm long, whilst the slow radial muscle-fibres are only about 100 μ long.

Fig. 2, A shows a transverse section (osmic-phosphotungstic fixation) of part of the highly folded muscle-sheet which constitutes the retractor muscle

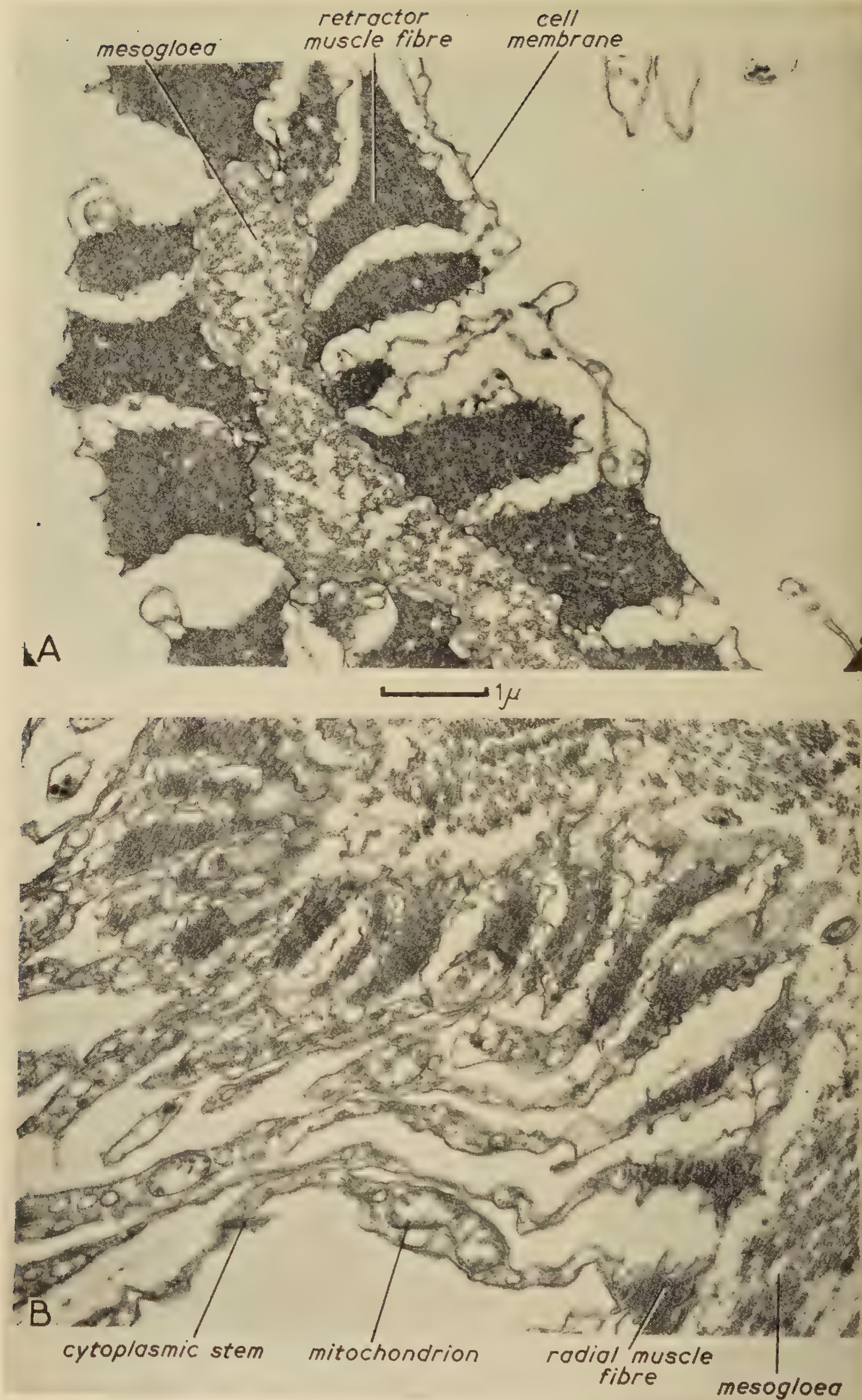

FIG. 2

A. V. GRIMSTONE, R. W. HORNE, C. F. A. PANTIN, *and* E. A. ROBSON

'he muscle-fibres are well defined and form an almost continuous layer over ne mesogloeal surface. Indeed, occasional lateral contacts of the cell memranes between the muscle-fibres suggest that there is some shrinkage in xation and that in this muscle contiguous fibres form a complete covering to ne mesogloeal surface.

In material fixed with osmic alone, adjacent muscle-fibres are often in close ontact. And though formolphosphotungstic fixes imperfectly, with this xation also the cell membranes of adjacent muscle-fibres are commonly in lose contact. This apparent close packing of the moderately extended nuscle-field contrasts with the open network of fibres seen in whole mounts f fully stretched actinozoan muscle-fields; fig. 2, A may be contrasted with late 2 of Batham and Pantin (1951). Robson (1957) noted that it might be ossible for small cytoplasmic processes from the base of the muscle-cells to rovide some attachment between adjacent fibres. Batham and Pantin's evience that the muscle-field was not a syncytium is reinforced by electron nicroscope preparations which give many examples of contact but not of yncytial connexion.

The sarcoplasmic crests rising from muscle-fibres are seen in transverse ection in fig. 2, A. The crests lie freely in the intercellular space and are ounded by the well-defined cell membrane. Under high magnification the ntire cell is seen to be bounded by such a cell membrane about 80 Å thick vhich, when viewed in strictly transverse section, is clearly double (figs. 4, B; , A; 7, D). A variety of methods used on a variety of other cells indicate that he cell membrane is double, about 75 Å thick (Rothschild, 1958). The cytolasmic crests are often somewhat empty of inclusions, except mitochondria figs. 2, A; 5 F). The appearance of the cytoplasm and mitochondria reembles that illustrated by Hess and others (1957) in the musculo-epithelial ells of *Hydra*. Occasionally mitochondria seem to be embedded in the subtance of the muscle-fibre.

The mitochondria are spherical or somewhat elongated and, as in fig. 5, F, hey are commonly about 0·3 to 0·5 μ in diameter. The walls are about 160 Å hick and seem double. In cross-section there are some 5 or 10 cristae, which n favourable sections seem to arise from the inner lamina of the wall and hemselves to consist of two laminae separated by 120 Å. No mitochondria lensely packed with cristae or with internal tubular structures were seen. The cristae are fewer in *Metridium* than in mitochondria in the higher animals cf. Sjöstrand and Hanzon, 1954*a*).

In contrast with the general cytoplasm of the epithelial part of the cell, the nuscle-fibre consists of a dense mass of longitudinal filaments. This clearly

FIG. 2 (plate). Osmic-phosphotungstic fixation, same scale for A and B.

A, transverse section of fold of moderately extended retractor (as in fig. 1, A (*b*) and B (*b*)). Note: cell membranes, mitochondria, filaments of muscle-fibres and of mesogloea in crossection, boundary layer of mesogloea.

B, transverse section of extended radial muscle-sheet of mesentery (as in (*c*) of fig. 1, A, B). Note smaller muscle-fibres, but with similar filamentous structure to that of A, and cytoplasmic tems leading to epithelial part of cell.

corresponds to the densely staining substance of the muscle-fibre described by Batham and Pantin (1951). Using the light microscope, they noted that the fibre sometimes gives the impression of consisting of two halves or fibrils separated by a core of less dense material. No evidence of this was obtained in our present observations and the appearance is doubtless an optical artifact.

In transverse section, the muscle-fibre seems clearly composed of filaments (figs. 4, C, D, E). They are shown by all methods of fixation, though their diameter varies with the fixative. In an extended muscle fixed with osmic-phosphotungstic acid they seem between 40 and 80 Å across. Despite the great contractility of these muscles the filaments of shortened fibres do not seem much thicker than extended ones.

In longitudinal section the filaments of extended fibres seem even thinner (30–40 Å) than in cross-section. Hanson (1957) gives 50 Å for the thickness of the filaments of earthworm muscle. The filaments in *Metridium* show no gross periodic structure comparable to that of striated muscle or indeed of some kinds of smooth muscle. The absence of this is evident in fig. 4, B. But near the limit of resolution available to us, the extended filaments in thin osmic-phosphotungstic sections such as this do show a rather irregularly beaded appearance with an interval of about 240 Å. There seems to be a tendency for the fibres to adhere laterally at these beads. Several fibres connected in this way may give some slight appearance of a transverse banding locally.

However, longitudinal sections are not always easy to interpret. The appearance naturally depends upon the precise angle of the section as well as on the fixation. It also depends upon whether the muscle is in a contracted or relaxed state at any length between the wide limits of the fully extended and the fully shortened muscle.

At all lengths, when in a state of contraction, the muscle seems composed of parallel filaments. But if the muscle is not in a state of tension, as shown by its buckled appearance in the whole section, it has the appearance of a network. Fig. 3 shows both well-extended muscle and fully shortened muscle (one-fifth extended length), in each case in the tensile state and in the relaxed. Examination of longitudinal sections of muscle under tension, particularly at full extension, suggest that this appearance of a network is due to adhesion of relatively long filaments, and is not due to a truly anastomosing meshwork. This is borne out by examination of formalin-fixed sections; even though the fixation in this case seems defective compared with osmium fixation. In transverse section the filaments clump together, whilst in longitudinal section the filaments adhere into an irregular number of bundles 1–2 μ long. But though the whole appearance and comparison with osmic and osmic-phosphotungstic

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FIG. 3 (plate). Longitudinal sections of retractor muscle. All osmic-phosphotungstic fixation. All same magnification.

A, extended and under tension. Note fine filamentous structure.

B, extended but relaxed. Note network appearance.

C, shortened to about one-fifth extended length, under tension. Note filamentous structure. Filaments apparently thicker than in A.

D, shortened but relaxed. Note network appearance.

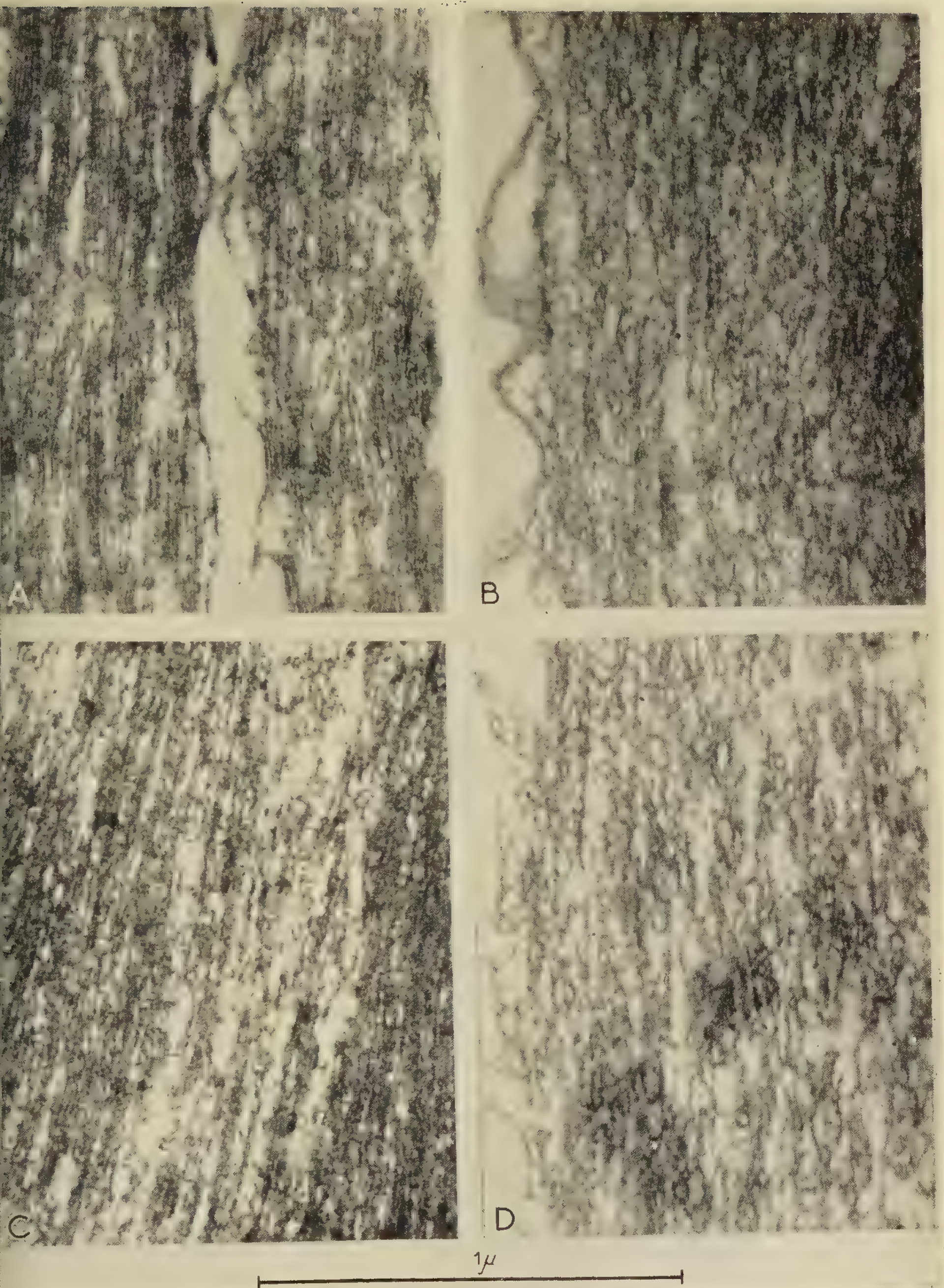

FIG. 3

A. V. GRIMSTONE, R. W. HORNE, C. F. A. PANTIN, *and* E. A. ROBSON

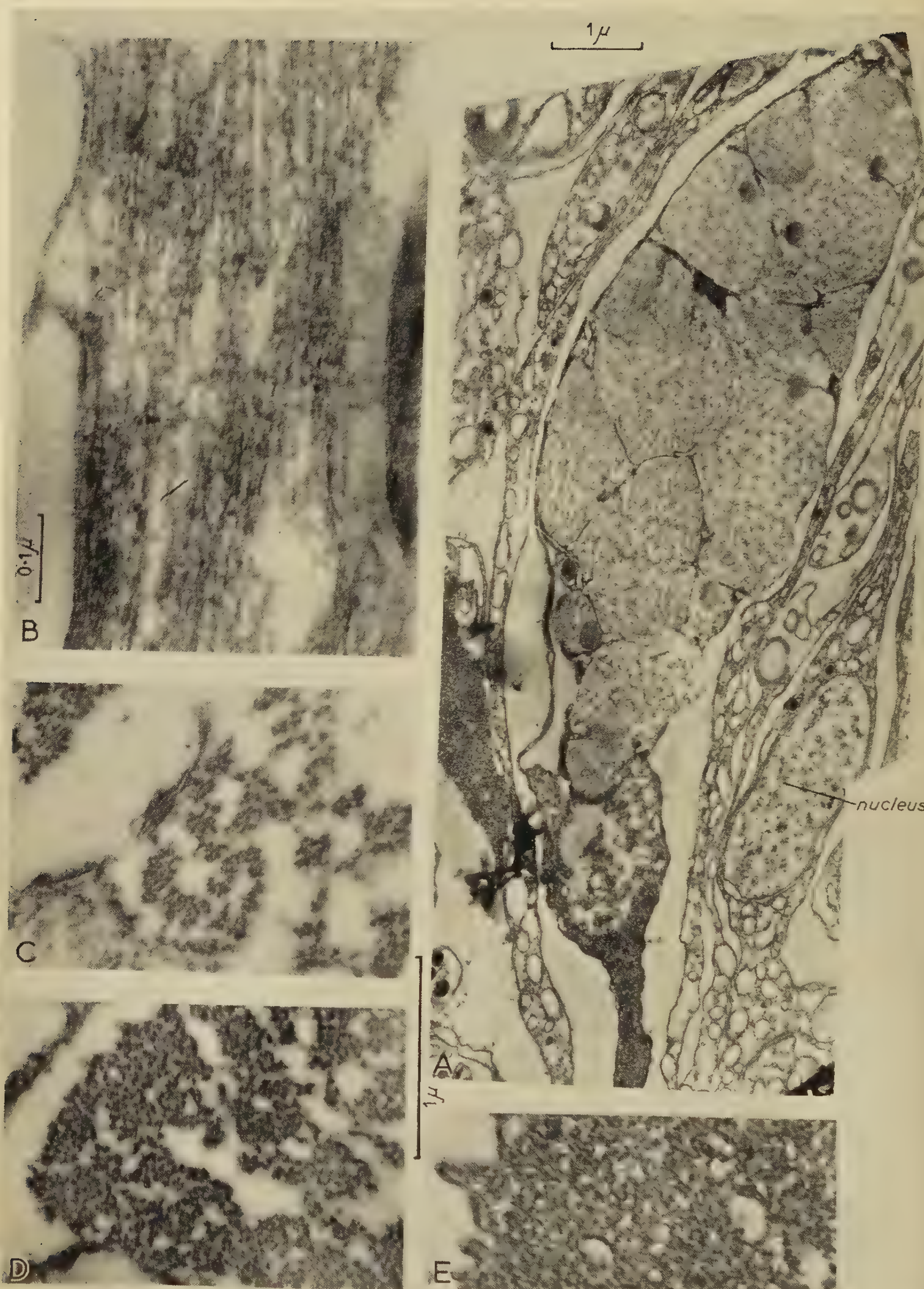

FIG. 4

A. V. GRIMSTONE, R. W. HORNE, C. F. A. PANTIN, *and* E. A. ROBSON

fixation shows that these long bundles are artifacts, their formation suggests that the filaments whatever their length are only connected firmly with each other at distances of several μ.

Radial muscle-fibres and cytoplasm

Fig. 2, B shows a transverse section of the muscle-field of the radial face of a perfect mesentery (fig. 1, A (*c*)). It may be compared with that of the retractor face as shown in fig. 2, A. When there is incomplete longitudinal extension of the mesentery the transverse muscle-field is thrown into folds at intervals of about 20 μ—the 'buckling' of Batham and Pantin (1951). The curve of the muscle-field in fig. 2, B is due to such a fold.

Functionally the radial muscle differs from the retractor in that it is much weaker and that it only contracts slowly. Fig. 2, A, B shows, however, that the general structure is essentially the same in both muscles except for the much smaller development of the muscle-fibre in the radial muscle-cells. The filaments are fewer in number than in the retractor, but their diameter (about 60 Å) and their packing are similar in the fibres of both muscles.

In both radial and retractor muscle the contractile filamentous material is wholly or almost wholly confined to the muscle-fibre. Robson has pointed out that the stems between the fibres and the epithelium are elastic and possibly contractile and follow changes of cell shape on extension of the muscle-sheet. Our preparations show the cytoplasm of the stems as well as of the crests to be rather empty of contents apart from mitochondria and vacuoles. The cell membrane, which is clearly defined, does sometimes show indications of fibrils in contact with it, and occasionally small amounts of fibrillar material somewhat resembling muscle-filaments can be traced into the epithelial stems. But such fibrillar material is always small in amount. The shortening of the surface of the epithelial stems which necessarily takes place when the muscle-fibres extend would seem operated by contractility or elasticity in the cell membrane itself.

The epithelium

As Robson (1957) showed, each stem arising from the cytoplasmic crest of the endodermal muscle-fibre passes up to a nucleated epithelial portion of the cell, bearing a flagellum. These same features are seen in detail in electron micrographs. The stems on the long thin epithelial parts of the cells carry nuclei (fig. 4, A). Some sections show a prominent nucleolus, but perhaps

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FIG. 4 (plate). Figs. C, D, and E are on the same scale.

A, osmic-phosphotungstic fixation. Transverse section of epithelium over retractor muscle-sheet (plane of fig. 1, C (*b*)) showing mucus-cell lying in intercellular space between stems of musculo-epithelial cells.

B, osmic-phosphotungstic fixation. Longitudinal section of part of extended retractor muscle-fibre in tension. Note filaments and tendency to beading at about 240Å intervals.

C, formol-phosphotungstic fixation. Transverse section of retractor muscle-fibre. Filaments partially dispersed and showing interfilamentar bridges.

D, formol-phosphotungstic fixation. Transverse section of retractor muscle-fibre to show partly ordered packing.

E, osmic-phosphotungstic fixation. Transverse section of retractor muscle-fibre at 50% extended length, partly relaxed, showing muscle filaments.

owing to imperfect fixation of the contents, the nuclei show little other organized structure in our preparations.

The cytoplasm, on the other hand, seems well fixed in both osmic and osmic-phosphotungstic preparations, though there may be some separation of the individual cells through shrinkage. Fig. 5, A, G, of the epithelial surface of the radial face of a perfect mesentery, shows the numerous vacuolar structures set in the cytoplasmic matrix. Some of these can be identified as mitochondria. There are also:

(1) other smaller vacuoles and laminate membranes such as may be seen at the bottom of fig. 5, G below the root of the flagellum. Presumably these represent the Golgi region (Sjöstrand and Hanzon 1954 *b*);
(2) densely staining bodies within a vacuole (fig. 5, H);
(3) thick-walled vacuoles (fig. 4, A).

The surface of the epithelial part of the cell is exposed to the gastric cavity. It appears naked, and is thrown into a remarkable series of membranes seen as free processes in all the preparations (fig. 5, G). There seems no reason to suppose that these are artifacts. They recall similar villous processes illustrated by Hess and others (1957) in the endodermal cells of *Hydra*.

Each cell bears a typical flagellum, with the usual 9 : 2 fibrous structure (figs. 5, B, C, D, E). After osmic-phosphotungstic staining the fibres appear to consist of pale rods surrounded by electron-dense material. Each outer fibre contains two such rods. In the inner pair, each contains one rod, so that this pair resembles somewhat one of the double-rodded fibres of the outer ring (fig. 5, B, C, D, E).

The flagellar membrane is continuous with that of the cell and small vacuoles occur between this and the fibres. The outer filaments run continuously into a densely staining basal corpuscle, corresponding to that seen with the light microscope. From this a fibrous root with marked striations (at

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FIG. 5 (plate). All osmic-phosphotungstic fixation.

A, longitudinal section through flagellum arising from crater on surface epithelium of radial muscle face. Note basal body and banded fibrous root, flagellar 'crater', also flagellar fibres and vesicles within cytoplasmic sheath of flagellum (plane of fig. 1, C (*b*)).

B, C, transverse sections of flagella. Note 9 : 2 fibre pattern, cell membrane, vesicles.

D, E, oblique section of base of flagellum and transverse section. Both enlarged from G. Note 9+2 fibre pattern and clear inner rod of each fibre. Outer fibres are double, each with two clear rods. Note vesicles.

F, horizontal section (as in fig. 1, A (*a*)) of retractor muscle-fibre, half extended; showing right muscle-fibre of musculo-epithelial cell with left mitochondria in cytoplasmic crest.

G, section in plane of fig. 1, C (*b*) through surface epithelium over radial muscle-sheet. Note flagellum rising from crater, basal body cut tangentially, membranes below root of flagellum. Note also cytoplasmic villi at epithelial surface, thickened cell membranes with intercellular bridges between outer parts of epithelial cells.

H, epithelial section, same place as D. Junction between epithelial cells. Note double cell membranes, thickened distally with intercellular bridges between outer components of membrane. Note that where the cell membranes are cut tangentially (near the surface and near the right side of the picture) the bridges appear as bars parallel to the epithelial surface. Note two mitochondria (bottom right).

Fig. 5

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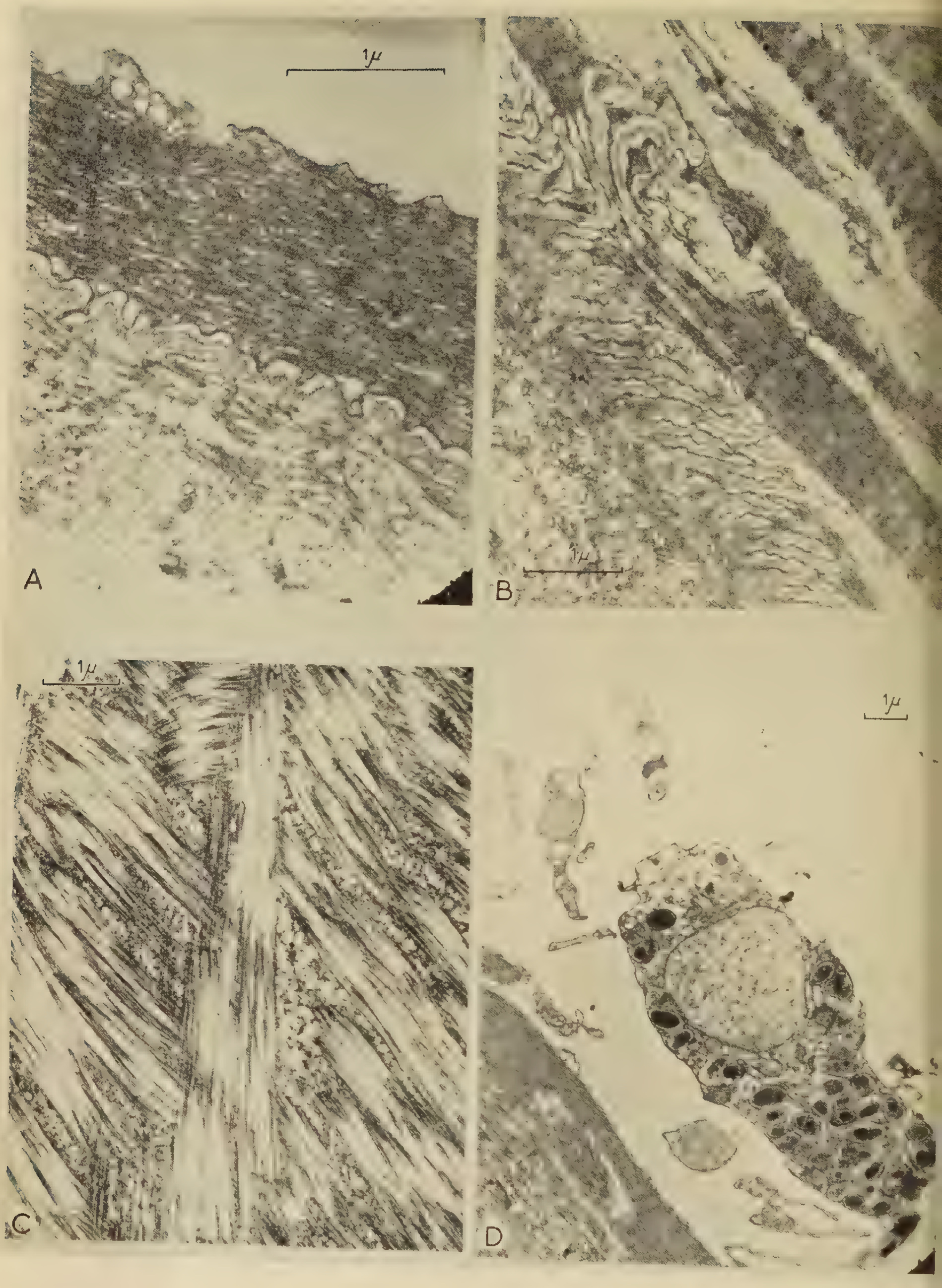

FIG. 6

A. V. GRIMSTONE, R. W. HORNE, C. F. A. PANTIN, *and* E. A. ROBSON

about 520 Å) passes down into the cytoplasm. At present we have no evidence for more than one root.

At the surface each flagellum is surrounded by a cytoplasmic 'crater' (figs. [illegible], A, G). Sometimes fine strands run between the crater and the base of the flagellum just before it enters the body of the cell.

The cell membranes of each cell are well defined. They are about 80 Å thick and appear double. Within about 0·5 μ of the outer surface of the epithelium the stained cell membranes become much denser to electrons. In this outer region there are regular ladder-like cross-connexions between the membranes of adjacent cells (fig. 5, G, H). Where the cell membrane is cut almost tangentially these cross-connexions appear as elongated bars parallel to the epithelial surface, rather than isolated pegs.

The densely staining outer region of the cell membrane may correspond to the cell boundaries which Robson has illustrated in stained preparations (Robson, 1957, fig. 1, F), and may perhaps be taken to represent secreted 'intercellular matrix'. Possibly also the 'basal granules' of Robson's light microscope figure may represent the basal craters of the flagella.

Mucus-cells and amoebocytes

In addition to musculo-epithelial cells, the general surface of the mesentery contains mucus-cells and amoebocytes. The region of the 'mesenterial filament' with its abundant cnidae and digestive and absorptive cells (Stephenson, 1928) was not examined by us.

Fig. 4, A shows a longitudinal section through a mucus-cell lying in the epithelium. The histological appearance easily enables it to be identified with such cells seen in fixed preparations under the light microscope. The ill-fixed nucleus is seen lying above the granular basal part of the cell which tapers downwards; whilst above are seen numerous mucus vacuoles in varying degree of dispersion about a central granule. Their appearance recalls that of the goblet cells of the intestine of vertebrates (Florey, 1955).

Amoebocytes are common in the immediate neighbourhood of the muscle-sheet, both in the intercellular space (fig. 6, D) above and in the mesogloea just below. There is no evidence that the amoebocytes secrete material in the mesogloea in any of our preparations, and a remarkable feature of the cells is their naked appearance.

That cells like those shown in fig. 6, D are the amoebocytes can be inferred

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FIG. 6 (plate). A, osmic-phosphotungstic fixation. Approximately vertical section along retractor muscle-fibre, 50% contracted. Note contact with basement layer of mesogloea, partially buckled.

B, osmic-phosphotungstic fixation. Almost horizontal section in plane of muscle (as in fig. 1, A (*a*) and C (*a*)). Muscle-fibres contracted to about one-fifth of extended length. Note extreme buckling of attachment layer of mesogloea with folds at about 0·25 μ.

C, formol-phosphotungstic fixation. Mesogloea. Note warp and woof structure, darkly stained amorphous material.

D, osmic-phosphotungstic fixation. Horizontal section through retractor (fig. 1, A (*a*) and C (*a*)), showing amoebocyte in the intercellular space. Note dark granules, and Golgi membranes above nucleus.

from their position and varied shape, from the absence of alternative objects with which they could be identified, and from their cytological appearance. Like the amoebocytes seen under the light microscope, these wandering cells are highly granular. The granules stain deeply, both with the two modes of osmic fixation and with the formalin-phosphotungstic method. In histological preparations under the light microscope granules of the same order of size are seen which stain strongly with acid fuchsin in Mallory's triple stain. Above the nucleus in the figure may be seen a system of canals or membranes which may represent Golgi membranes.

At the magnification employed, sensory cells and nerve-axons are few and far between (Pantin, 1952). The probability of a cut axon appearing in one of the sections is low, and we found no object that at present could certainly be identified as one.

Mesogloea

That the mesogloea has an organized fibrous structure of collagen is known from examination of its structure and from its mechanical properties (Chapman, 1953). On both grounds Batham and Pantin (1951) showed that there must be an outer, moderately extensible, portion of the mesogloea to which the muscle-fibres are attached. Fig. 6, A shows clearly the reality of Batham and Pantin's mesogloeal basement membrane to which the muscles are attached. It is a three-dimensional network attached directly to the cell membrane of the muscle-fibre above and it passes rather suddenly, but continuously, into the grosser mesogloeal fibres below. It follows the normal buckling of the muscle-sheet with folds at intervals of the order of 20 μ, already described. But this superficial mesogloeal layer also carries folds of the muscle-fibre and its cell membrane. These folds project as pockets into the mesogloea at intervals of the order of 0·2 to 0·3 μ. That this is not a fixation artifact is shown both by the relation of these folds to the fibrous structure of the underlying mesogloea and of the muscle, and also by the way they increase with the contraction of the muscle. Fig. 6, B shows the extreme folding of this layer of mesogloea and the muscle-cell membrane under maximal muscular contraction. It is clear that this basement mesogloeal layer and the folds into which it is thrown during contraction provide a firm attachment of the muscle-fibre through its cell wall.

The structure of the fibres of Chapman's mesogloeal fibrous lattice, which composes the bulk of the mesogloea, is of particular interest. Whilst sections show some amorphous fine material in the general mesogloeal spaces, most of the solid material is made up of clusters (figs. 6, C; 7, A) of fibrils. The fibres which make up Chapman's cross-lattice are evidently these clusters of fibrils. Under high resolution it is, however, seen that the clusters are not well-defined entities; fibrils pass from one cluster to another of the lattice.

The fibrils themselves, far beyond optical resolution, have well-defined structure and are of regular size (fig. 7, A. C). Their diameter varies slightly about 100 Å and they show a characteristic pattern of periodic bands spaced

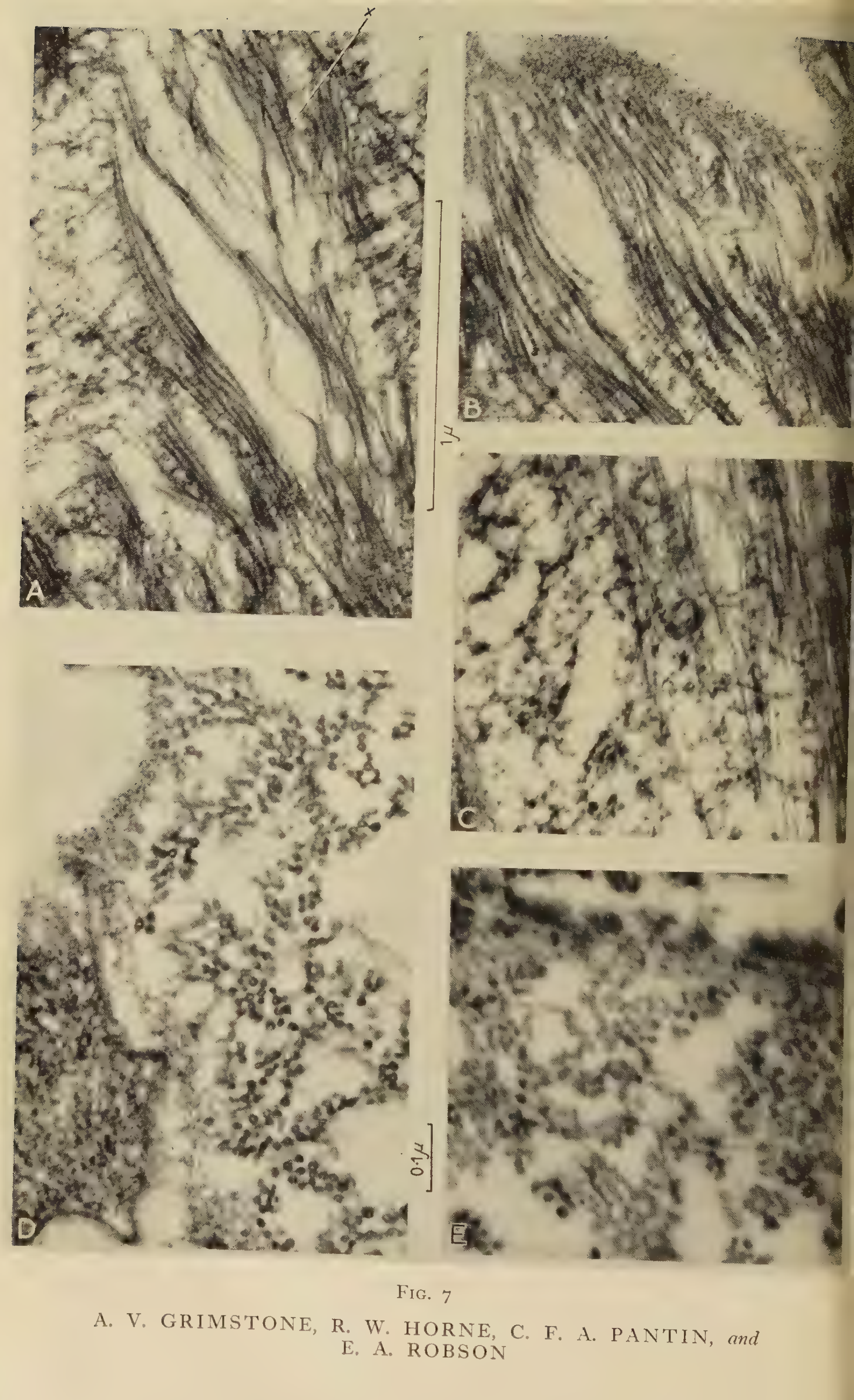

FIG. 7

A. V. GRIMSTONE, R. W. HORNE, C. F. A. PANTIN, *and*
E. A. ROBSON

at about 260 Å. In osmic-phosphotungstic preparations these fibrils often give the appearance of being hollow tubes. This is strikingly the case when well-focused fibrils are seen precisely in cross-section (fig. 7, D).

The fibrils are easily seen after simple osmic fixation though the contrast is not sufficient to show certainly whether these seem tubular. The fibrils seen in the formalin-phosphotungstic preparations have about the same dimensions and the characteristic banding at about the same interval (figs. 6, C; 7, D, E) as in osmic-phosphotungstic preparations.

With all three methods of fixation there is, in addition to the fibrils, some amorphous substance. Its character and quantity differ somewhat with each fixative, so that there is some reason to suppose that it is an artifact of fixation (compare figs. 7, A, C). The amount of amorphous material not organized into fibrils appears least after simple osmic fixation.

But the fibrils survive all three methods of fixation, and do so in similar form and relation. Where contrast is sufficient for detail to be seen, as in both osmic-phosphotungstic and in formalin-phosphotungstic preparations, the fibrils are seen to adhere in bundles along part of their length to give the fibres of Chapman's lattice. As figs. 6, C and 7, A show, when they do so, the periodic banding extends through the whole bundle—the fibrils aligning themselves so that their banding corresponds to that of its neighbours.

Discussion

Before accepting electron micrographs as evidence of structure in living cells, two classes of error must be considered. We must show that the appearance we examine is not of instrumental origin, as in optical diffraction or interference; and we must show that it is not an artifact of fixation or subsequent treatment. In the light microscope the appearance of double membranes at cell surfaces or the tubular appearance of mesogloeal fibrils might raise such suspicion of a diffraction effect. But considering the dimensions of the instrument we used and the exceedingly short equivalent wavelength of the electron beam (about 0·041 Å) it becomes clear that we cannot attribute these appearances in the structures we are considering to an effect analogous to optical diffraction. Effects analogous to optical interference are less easy to dismiss, and, as is well known, are sometimes apparent in electron micrographs. There is, however, a test which can be applied. False images of instrumental origin will vary with instrumental conditions, that is with the applied magnification

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FIG. 7 (plate). B and C are on same scale as A. D is on the same scale as E.

A, osmic-phosphotungstic fixation. Note co-ordinated banding of fibril-bundles (at x); also amorphous material.

B, osmic-phosphotungstic fixation. Tangential section of mesogloea showing passage of fibrils into surface layer of mesogloea.

C, formalin-phosphotungstic fixation. Mesogloea, showing fibres and amorphous material.

D, osmic-phosphotungstic fixation. Transverse section through retractor (plane of fig. 1, A (b)). Note filaments of muscle-fibres, cell membrane, mesogloeal attachment layer, apparently tubular mesogloeal fibrils.

E, formol-phosphotungstic fixation. Transverse section of retractor. Note apparently tubular mesogloeal filaments as in C.

and with the focus; and such false images will be apparent in all objects of the same dimensions in the object examined. Now the dimensions of the features of the cell membranes and of the mesogloeal fibrils which we are discussing are independent of magnification by the instrument and of the focus. Moreover, the appearance of double structure is quite evidently absent in many minute features in micrographs which clearly illustrate it in the cell membrane; whilst a tubular appearance like that of the mesogloeal filament is absent in the cross-section of other filaments and of granules of the same size, and in the same micrograph. We may therefore conclude that these features are not instrumental artifacts.

The possibility of histological artifact is still more difficult to eliminate. Grosser features can be directly identified with structures seen optically in the living cell, but finer detail cannot receive this direct confirmation. However, there is strong circumstantial evidence for the reality of some electron-micrographic structure in the living cell. The fact that the mesogloeal fibres are seen after both osmic and formol fixation lends probability to the supposition that the appearance is not an artifact. Whether they are hollow tubes, as they appear to be after phosphotungstic treatment, or whether for some reason the cortex of the fibres takes stains differentially, cannot at present be determined; though we may note that the very different method of metallic shadowing suggests that the rather similar fibres of vertebrate collagen may be tubes (Wyckoff, 1952).

Further evidence for the reality in life of electron-micrographic structures may be gained by what may be termed 'the principle of functional connexions'. If some feature of a structure seen in an electron micrograph fulfils, and alone fulfils, an evident functional requirement inferred on other grounds there is presumptive evidence for its existence in life. Thus the known contractility of the muscle-fibres implies the existence of contractile structures in them which are in turn attached, directly or indirectly, to the underlying mesogloea. The muscle filaments seen under the electron microscope fulfil this requirement and there is no other evident structure which could do so. On the other hand, any feature that seems attributable to the histological treatment which the specimen has received is a presumptive artifact. That is seen in shrinkage, disruption of membranes, or regional differences in fine structure correlated with the direction of penetration of histological reagents.

By these tests we can obtain quite strong likelihood for the existence in the living cell of structures inferred from electron micrographs. This is especially true if supporting evidence comes from sources of widely different character. Thus, the reality of the differentiated fibrous layer at the surface of the mesogloea and in contact with the muscle-fibres is strongly supported by the fact that Batham and Pantin (1951) predicted the existence of such a layer from the mechanical behaviour of the tissue.

Other kinds of evidence undoubtedly affect the subjective estimate of the observer. Of these the most important is the degree of organization of structure. Whether or not a functional significance can be attributed to it, a

structure that shows well-defined organization both generally and in detail creates a strong impression that it was present in the living cell from which the preparation is derived. It is difficult to justify this impression except on the ground that experience often justifies it. Nevertheless, it is important because the first detection of significant structure often proceeds directly from it; the other kinds of evidence being used to justify what is in fact a tentative subjective hypothesis.

Judged by these criteria, the structures described in this paper can with fair probability be taken to correspond to things in the living cell. We shall therefore briefly comment on them, making that assumption.

The epithelium

The epithelial part of the musculo-epithelial cells shows striking similarities to the ciliated epithelial cells described by Fawcett and Porter (1954) in lamellibranchs and vertebrates. The flagella of *Metridium* endoderm have the familiar 9 : 2 fibrous structure, though in *Metridium* the double nature of the outer fibres is so evident (fig. 5) that it might as easily be called an 18 : 2 structure. The fibres arise from a hollow basal corpuscle about 2,000 Å wide and 3,500 Å long. Unlike molluscan cilia, but like those of the ctenophore *Pleurobrachia pileus* (Bradfield, 1955), the fibres continue into the basal corpuscle. As in other cases a fibrous root, perhaps single in *Metridium*, passes from the basal granule into the cytoplasm, and this root shows well-marked striation at about 520 Å, which may be compared with 500 Å for the root of *Pleurobrachia* cilia. Goreau and Philpott (1956) describe similar structures in two madreporarian corals, though the periodicity of the striation in the root is given by them as 670 Å.

Like other flagella and cilia, the flagellum of *Metridium* is bounded by a membrane continuous with that of the cell proper. In *Metridium* small vesicles are seen in electron micrographs on one side of the flagellar fibres, particularly near its base. This is of some interest. The retinal rods in vertebrates arise as modifications of ciliated cells (Sjöstrand, 1956). The remarkable pile of membranous sacs of the retinal rod apparently arises from the growth and flattening of vesicles on one side of the ciliary fibres (de Robertis, 1956). The developmental stages of the vesicles described by de Robertis are by no means unlike the permanent state of *Metridium* flagella with their lateral vesicles. The musculo-epithelial system of *Metridium* has a general sensitivity to light and the action spectrum somewhat resembles that of vertebrate rods (North and Pantin, 1958). Flagella are known to be light-sensitive in some organisms (Mast, 1938). The possibility that this may be true of *Metridium* flagella warrants investigation.

The remarkable crater round the base of *Metridium* flagella and the membranes and cytoplasmic villi which project from the surface of the epithelium resemble the similar and more numerous epithelial and surface structures described in the ciliated epithelium of animals from several different phyla (Bradfield, 1955; Goreau and Philpott, 1956). In *Metridium* the presence of

these extensions of the cell membrane raises a new problem. The epithelial part of the cells undergoes very great extension and contraction according to the state of expansion and contraction of the animal. How can a villous cell surface of this kind accommodate itself to such extensive deformation? We are very far from being able to consider the epithelial surface as a simple 'surface-tension layer'. It must have reversible extensibility which, nevertheless, permits the retention of these surface features. This feature may be correlated with the peculiar intercellular surface structures of *Metridium* epithelio-muscular cells. In the deeper parts adjacent cells are each bounded by a double cell-membrane which may be in contact with that of its neighbour but which does not adhere to it. Indeed, between the epithelial and muscular parts of the cells the cytoplasm is narrowed to neck-like strands which are separated by intercellular fluid (Robson, 1957). But in the outermost region of the epithelium there is an electron-dense connecting membrane apparently holding the adjacent cells together. As elsewhere the cell membrane is double, though here it is deeply stained; but here and there the outer component of each cell membrane is connected to that of its neighbour by strands like the rungs of a ladder. 'Intercellular bridges' or desmosomes are well known in the tissues of other animals. But they are exceptionally strongly developed near the surface of *Metridium* epithelium. In vertebrate squamous epithelium and many other tissues, all that is to be seen are darkly staining masses in the cytoplasm at corresponding points beneath the cell surface (Fawcett and Selby, 1958). Adjacent cell surfaces are separated by a clear space of 150–200 Å without visible structure. In *Metridium*, whilst there are such dark masses below the cell membrane, in addition bridges actually connect the outer surfaces of the adjacent cell membrane (fig. 5, H). Well-developed cell boundaries would certainly seem a necessity in highly extensible epithelium.

Muscle

Striated muscle from a variety of animals seems to consist of two kinds of filaments, thick myosin filaments in the A band and thinner actin filaments extending through the I band and into the A band. According to a current hypothesis the contraction of such muscles takes place in effect by the sliding of the actin filaments along the spaces between myosin filaments (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). A cardinal feature of this hypothesis is necessarily the presence of two sorts of filaments. Recently Hanson and Lowy (1957 *a*, *b*) have shown that a double array of filaments comparable to that of striated muscle occurs in a variety of unstriated muscles from various animals; though in these the two kinds of filament occur throughout the length of the muscle and are not aggregated in gross striae along its length. They suggest that a similar sliding mechanism between the two sorts of filament may account for contraction in plain and in striated muscle.

Both the cytological and ultimate filamentous structure of the muscle-fibres of *Metridium* seem rather simpler than those of unstriated muscles so far described, except perhaps for that of the unstriated muscle of the frog's

adder (Hanson and Lowy, 1957 *b*). Though there is some tendency for gularity of packing of the muscle filaments (fig. 4, C, D), there is none of the gular organization of fine structure seen in some of Hanson and Lowy's olluscan and annelidan unstriated muscles. Nor do they show clear evidence more than one kind of filament; though these often seem connected by intertial bridges of other material (fig. 4, C). Clearly, the nature of this interstitial aterial needs further investigation, but such preparations as we have made far show only one sort of clearly defined filament.

Whether or not this interstitial material disguises some second kind of filaent such as Hanson and Lowy find in molluscs, it seems certain that *Metriium* muscle is physiologically of the same class as other sorts of muscle. Not

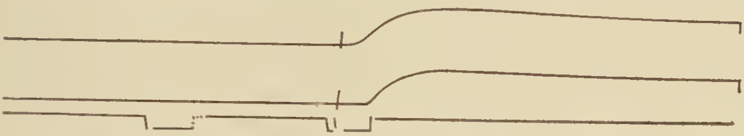

FIG. 8. Facilitated retractor response of *Metridium* to two condenser shocks at 1-sec. interval. Recorded from two opposite points on the disk. Note rapid development of tension. Recorded by a light spring lever.

nly are the mechanical properties of the muscle essentially the same (Batham nd Pantin, 1950 *a*) but actomyosin-like proteins which can be caused to conact by ATP have been extracted from the actinian *Anthopleura japonica* Maruyama, 1956).

In comparing the functional organization of an unstriated muscle like that f *Metridium* retractor with that of a vertebrate striated muscle, there is one ther point that is perhaps worth bearing in mind. It is still often considered at the chief functional distinction between striated and unstriated muscle is at the latter contracts more slowly than the former. But this distinction is ot reliable (Pantin, 1956), and Hanson and Lowy (1957 *b*) point out that the gnificance of cross-striation is still unexplained. However, certain clear ropositions can be made which current hypotheses of muscular contraction o not seem fully to take into account (Pantin, 1956). These are:

(1) Some unstriated muscle can develop tension as swiftly as many unstriated muscles. Despite its repeated evolution in different animals striation is not thus a necessary feature of swift contraction. Fig. 8 shows the response of *Metridium* retractor working under more or less isometric conditions. The contraction phase reaches 50% of the maximum tension in about 0·1 sec whilst maximum tension is often developed in much less than 0·3 to 0·5 sec. Under rigidly isometric conditions unobtainable in *Metridium* the development of maximum tension would be even more rapid. Some other unstriated muscles can contract even faster.

(2) Unstriated muscle has an enormous range of resting length. Fully extended resting retractor muscle in *Metridium* is more than 5 times as

long as the resting fully shortened muscle. For the longitudinal muscles of *Hydra* tentacles the factor is not 5 but 20.

(3) Compared with striated muscle, all unstriated muscles relax much more slowly then they contract.

These propositions suggest that the development of striation is related to speed and extent of relaxation rather than to speed of contraction: yet none of them seems to arise as a necessary consequence of current hypotheses about the functional significance of striation in muscle.

Mesogloea

Knowledge of the mesogloeal proteins of actinians is at present limited, but there is reason to suppose that they are related to collagen. Chapman (1953) showed many resemblances between actinian mesogloea and vertebrate collagen, histologically, chemically, and in physical properties. He also drew similar conclusions about the mesogloeal fibrous matter of various medusae. That is in agreement with X-ray diffraction studies by various authors of the mesogloea of medusae and Actinozoa (Rudall, 1955). But Bouillon and Vandermeerssche (1956) have concluded that the fibres in medusae are not collagen but elastin. Whether that is so or not—and the authors differ on certain questions of fact—it seems probable that Chapman is right in supposing the mesogloeal fibres of actinians to be a collagen because of the chemical composition, particularly the abundance of proline and hydroxyproline, and because of the contraction under heat and the solubility in dilute acids. Lenhoff and others (1957) record hydroxyproline in collagen fibres from *Metridium*.

Like collagen, the ultimate fibrils of the mesogloeal substance show regular banding. The period, which varies slightly with the preparation, ranges from 220 Å to 250 Å. Comparable periods are known from some vertebrate connective tissue (Wyckoff, 1952), but the characteristic banding of mature collagen at about 640 Å has not been found by us in the mesogloeal fibrils. But other periodicities occur in collagen, as in the 520 Å period in the basement membrane of amphibian larvae (Weiss and Ferris, 1956) and at 210 Å in immature connective tissue from chick embryos (Porter, 1951). In mesogloea from the southern species *M. canum* Dr. E. J. Batham has preparations showing accentuated banding of 440 Å.

Whatever the ultimate nature of the mesogloeal fibrils, their organization into higher units is very like that of vertebrate connective tissue. The fibrils are grouped into fibres and bands as in Wyckoff's (1952) figure for collagen fibres in chick tendon.

Chapman (1953) showed the striking crossed lattice organization of the fibre aggregates in the mesogloea of *Calliactis* and called attention to the great importance of this for the extensibility of the tissue. Weiss (1957) records what seems a quite parallel organization in the basement membrane of amphibian larvae.

The question arises how this crossed lattice pattern originates. The evidence both in vertebrates and in coelenterates suggests that the fibrils arise as extra-

cellular exudates and are only secondarily organized into fibres of the connective tissue lattice (Bretschneider, 1951); Chapman, 1953; Weiss, 1957; Jackson and Smith, 1955). Chapman points out that crossed lattices are a common feature of systems exposed to stresses. Our own observations on the fine structure of the lattice support the idea that the organized aggregation of the fibrils is imposed on the fibrils, for under the electron microscope the fibre-aggregates are seen not to be well-defined structures; individual fibrils pass freely from one fibre-bundle to another.

It is an essential feature of any such process of aggregation that to constitute fibres the fibrils must have the power to adhere to each other, and this is clearly seen in the manner in which the fibrils in *Metridium* mesogloea aggregate band by band—just as Weiss notes in amphibian basement membrane.

This still leaves the ultimate problem unsolved of how the fibre-bundles of the lattice come to be arranged in alternating layers obliquely to the axis of the animal, as Chapman has shown. It is not enough to point out that once it has arisen such a system, and such a system alone, allows the necessary flexibility of the tissues. Possibly some kind of 'natural selection' during the normal muscular movements, longitudinal and transverse, may operate. Adhering groups of fibres along an axis may tend to be parted during extension along that axis; whilst obliquely arranged aggregates might survive through their lattice-like freedom of movement relative to the axis.

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